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(54) Title: CYTOPLASMIC ANTIPROTEINASE-3 CODING SEQUENCE IN GENE THERAPY

(57) Abstract

Cytoplasmic antiproteinase-3 nucleic acids and serine and cysteine protease inhibitor proteins encoded thereby useful in the purification of proteins and in the treatment of inflammatory diseases and diseases involving apoptosis are provided.

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CYTOPLASMIC ANTIPROTEINASE-3 CODING SEQUENCE IN GENE THERAPY

INTRODUCTION

Field of the Invention

This invention relates to treating a disease or symptoms of a disease mediated by a caspase by transiently expressing a proteinaceaus anticaspase gene in a diseased target tissue. The invention is exemplified by the use of cytoplasmic antiproteinase-3 (CAP-3) to inhibit interleukin-1β-converting enzyme (ICE;caspase-1) and plasminogen activator inhibitor-2 (Ich-2;caspase-4).

Background

Cysteine proteases play a critical role in many physiological processes. For example, cysteine proteases are involved in blood coagulation, fibrinolysis, complement activation, and inflammation. The catalytic activity of these cysteine proteases and other protease families are often regulated by members of a super-family of cysteine protease inhibitors called serpins. Serpins act to regulate the activity of cysteine proteases by binding stoichiometrically to the active sites of cysteine proteases and thus inactivating the enzymes.

Many serpins are extracellular proteins which regulate extracellular processes such as blood coagulation, fibrinolysis and complement activation. In addition, there is a family of serpins structurally related to ovalbumin which lack secretory peptide sequences and which may function, in part, intracellularly. Several of this latter group of serpins are believed to play an important role in regulating cysteine protease activity in inflammation. Elastase inhibitor is an example of such a serpin which functions to regulate the activity of neutrophil elastase. Neutrophil elastase is stored in the azurophil granules of neutrophils, monocytes and macrophages, and degrades both phagocytized and extracellular substrates. Regulation of neutrophil elastase is important both in host defense mechanisms and also in the pathology of diseases such as arthritic joint diseases.

Interleukin-1β converting enzyme (ICE;caspase-1) is an example of a cysteine protease that plays an important role in inflammation. ICE is responsible for the activation of interleukin-1β, which is a critical cytokine in the inflammatory process. Other members of the ICE subfamily, caspases-4 and -5 also play a predominant role in inflammation. Serpins which

inhibit ICE may therefore play an important role in inhibiting inflammation. One such serpin is a viral protein encoded by the cowpox virus crmA gene. It is believed that expression of crmA protein inhibits ICE and thereby blocks migration of inflammatory cells in cowpox lesions (*see* Ray, *et al* (1992) *Cell* 69:597-604), thereby facilitating the viral infection. crmA also inhibits other caspases including caspase-8. Intracellular expression of serpins that inhibit ICE in a manner similar to the crmA protein can be useful in the modulation of the inflammatory response and used in the treatment of a variety of inflammatory diseases, such as rheumatoid arthritis.

ICE is but one member of a family of cysteine proteases that play important roles in normal physiology and in pathophysiology. (Iyer *et al*, *Toxicol. Appl. Pharmacol.* (1996) 141(1):84-92; Iyer *et al*, *Am. J. Physiol.* (1997) 273(4Ph1):L760-L767.) For example, another member of the ICE family, Ich-1 (caspase-2), is involved in regulation of apoptosis as are caspases-3 and -10. Other caspases that are involved in apoptosis include caspase-7, -6, -8, and -9 (Zhou *et al*, *J. Biol. Chem.* (1997) 272:7797-7800). Furthermore, evidence is accumulating that regulation of apoptosis plays a role in a variety of different diseases, including cancer. Therefore, intracellular expression of serpin molecules which inhibit Ich-1 could be used to regulate apoptosis and treat a number of diseases, including neurogenerative diseases such as Alzheimer's disease, Parkinson's disease, amylotrophic lateral sclerosis (ALS), and acute injury such as hypoxia-ischemia or trauma. Current treatment regimens for these diseases may slow progression of the disease process or offer temporary relief from disease symptoms, but do not prevent loss of neurons. Other diseases also implicate an apoptosis etiology and could be treated, including tumor necrosis factor induced acute-liver failure following toxin exposure, liver destruction in hepatitis B, cirrosis, endothelial cell hypoxia and reperfusion injury.

Isolated serpin molecules are useful in the purification of a variety of proteins for use in medicine and industry. Protein degradation during purification by endogenous cysteine proteases is a common problem. Isolation of serpins inhibiting different cysteine proteases is useful to improve the purification of many different proteins. These and other needs are addressed by the present invention.

Relevant Literature

Characterization of CAP-3 (PI9) is described in Sprecher *et al*, (1995) *J. Biol. Chem.* 270:29854-29861. Carrell *et al*, (1987) *Cold Spring Harbor Symposia*, vol. LII, pp. 527-535 provides a description of the structural characteristics of serpins. A review of the role of caspases in apoptosis appears in Nicholson and Thornberry, (1997) *TIBS* 22:299-306. Also see Bainaga, (1998) *Science* 280:32-34.

Small molecules that inhibit ICE are described in USPNs 5,416,013; 5,585,357; 5,565,430; 5,639,745; 5,677,283, and 5,552,400.

A large number of publications relate to *in vivo* and *ex vivo* transfection of mammals. The following are examples of the publications in this area.

A variety of approaches for introducing functional new genetic material into cells, both *in vitro* and *in vivo* have been attempted (Friedmann, (1989) *Science* 244:1275-1281). These approaches include integration of the gene to be expressed into modified retroviruses (Friedmann, (1989) *supra*; Rosenberg, (1991) *Cancer Research* 51(18), suppl.:5074S-5079S); integration into non-retrovirus vectors (Rosenfeld *et al*, (1992) *Cell* 68:143-155; Rosenfeld *et al*, (1991) *Science* 252:431-434); or delivery of a transgene linked to a heterologous promoter-enhancer element via liposomes (Friedmann, (1989), *supra*; Brigham *et al*, (1989) *Am. J. Med. Sci.* 298:278-281; Nabel *et al*, (1990) *Science* 249:1285-1288; Hazinski *et al*, (1991) *Am. J. Resp. Cell Molec. Biol.* 4:206-209; and Wang and Huang, (1987) *Proc. Natl. Acad. Sci. (USA)* 84:7851-7855); coupled to ligand-specific, cation-based transport systems (Wu and Wu, (1988) *J. Biol. Chem.* 263:14621-14624) or the use of naked DNA expression vectors (Nabel *et al*, (1990), *supra*); Wolff *et al*, (1990) *Science* 247:1465-1468). Direct injection of transgenes into tissue produces only localized expression (Rosenfeld, (1992) *supra*); Rosenfeld *et al*, (1991) *supra*; Brigham *et al*, (1989) *supra*; Nabel, (1990) *supra*; and Hazinski *et al*, (1991) *supra*). The Brigham *et al* group ((1989) *Am. J. Med. Sci.* 298:278-281 and (1991) *Clinical Research* 39 (abstract)) have reported *in vivo* transfection only of lungs of mice following either intravenous or intratracheal administration of a DNA liposome complex. An example of a review article of human gene therapy procedures is: Anderson, (1992) *Science* 256:808-813.

PCT/US90/01515 (Felgner *et al*) is directed to methods for delivering a gene coding for a pharmaceutical or immunogenic polypeptide to the interior of a cell of a vertebrate *in vivo*. Expression of the transgenes is limited to the tissue of injection. PCT/US90/05993 (Brigham)

is directed to a method for obtaining expression of a transgene in mammalian lung cells following either iv or intratracheal injection of an expression construct. PCT 89/02469 and PCT 90/06997 are directed to *ex vivo* gene therapy, which is limited to expressing a transgene in cells that can be taken out of the body such as lymphocytes. PCT 89/12109 is likewise directed to *ex vivo* gene therapy. PCT 90/12878 is directed to an enhancer which provides a high level of expression both in transformed cell lines and in transgenic mice using *ex vivo* transfection. PCT/US92/08806 is directed to particle-mediated transformation of mammalian unattached cells. EP application 91301819.8 is directed to the use of recombinant techniques to produce cystic fibrosis transmembrane conductance regulator (CFTR).

SUMMARY OF THE INVENTION

Methods are provided for treating a disease and/or disease symptoms mediated by a caspase by administering to a host a gene encoding an intracellular mammalian serpin. The method includes providing a non-integrating DNA construct which comprises an operably linked transcriptional promoter, a DNA segment encoding a polypeptide which inhibits serine or cysteine proteinase activity and an transcriptional terminator to an individual with a caspase-mediated disease or disease symptom so that the polypeptide is transiently expressed in a target tissue affected by the disease or symptoms of the disease. The transcription promoter and terminator are functional in the target tissue. Optionally the DNA construct is complexed with a cationic lipid carrier which facilitates uptake by the target tissue. The method finds use in the alleviation of disease or disease symptoms in the target tissue.

The present invention provides isolated nucleic acid molecules encoding mammalian CAP-3 protein. The CAP-3 protein of the invention is homologous to the amino acid sequence depicted in SEQ ID NO:4, including the amino acid sequence depicted in SEQ ID NO:4, or an allelic variant thereof. Typically the CAP-3 protein and polypeptides thereof will be capable of inhibiting cysteine protease activity. The isolated nucleic acid molecule, e.g., DNA or RNA, encode human CAP-3 protein.

Thus, in another aspect the invention includes isolated mammalian CAP-3 protein homologous to the amino acid sequence of SEQ ID NO:4 which inhibits cysteine protease activity. In exemplary embodiments described herein the CAP-3 proteins are human, e.g., the protein of SEQ ID NO:4, or an allelic variant thereof.

In other embodiments the invention provides expression vectors having as operably linked elements a transcriptional promoter, a DNA segment encoding a mammalian CAP-3 protein wherein said protein is homologous to the amino acid sequence depicted in SEQ ID NO:4 or an allelic variant, and inhibits cysteine protease activity, and a transcriptional terminator. Cultured host cells also are provided which are transformed or transfected with these expression vectors. Preferably the host cell is a mammalian cell.

In other aspects the invention provides methods for purifying a protein in a solution which contains a protease. The solution containing the protein of interest is exposed to a mammalian CAP-3 cysteine protease inhibitor, for example, that which has been immobilized to an affinity column, whereby the protease binds to the inhibitor and the activity of the protease is inhibited. One or more subsequent separation or purification steps can be performed in the presence of said cysteine protease inhibitor to obtain said purified protein.

In another embodiment methods are provided for producing a mammalian CAP-3 polypeptide by growing eukaryotic cells, especially mammalian cells, transformed or transfected with a DNA construct which comprises an operably linked transcriptional promoter, a DNA segment encoding a mammalian CAP-3 polypeptide which inhibits cysteine protease activity, and a transcriptional terminator. The CAP-3 polypeptide is preferably homologous to the amino acid sequence of SEQ ID No:4. The cells are cultured under conditions whereby the CAP-3 encoding DNA segment is expressed. The mammalian CAP-3 polypeptide can then be isolated from the host cells, e.g., by affinity purification or similar procedure.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 shows the effect of CAP-3 on substrate hydrolysis by caspases. Zero time corresponds to the addition of CAP-3. Fig. 1A shows progress curves for the hydrolysis of 20 μM AcYVAD-pNA by 1.5 nM ICE in the presence of (top to bottom) 0, 25 nM, 50 nM, 100 nM, 200 nM, or 500 nM CAP-3. Fig 1B shows progress curves for the hydrolysis of 20 μM AcDEVD-pNA by 0.15 nM CPP32 in the absence (solid curve) or presence (dashed curve) of 500 nM CAP-3. Fig 1C shows progress curves for the hydrolysis of 20 μM AcYVAD-pNA by 50 nM Ich-2 in the absence (top curve) or presence (bottom curve) of 100 nM CAP-3. Fig 1D shows the data from Fig. 1A were fit to equation (1), and the apparent first-order

inactivation rate constant is shown as a function of CAP-3 concentration, demonstrating an apparently linear relationship.

Fig. 2 shows the effect of substrate concentration on the inhibition of caspases by CAP-3. Substrate (20 or 200 μM AcYVAD-pNA) was hydrolyzed by 5 nM ICE (Fig. 2A) or 50 nM Ich-2 (Fig. 2B) in the presence (dashed lines) or absence (solid lines) of 100 nM CAP-3. Fig. 2C shows the variation with substrate concentration of the apparent rate constant for inactivation of ICE in the presence (O) or absence (□) of 500 nM CAP-3.

Fig. 3 shows the inactivation of caspases by CAP-3 in the absence of substrate. Fig. 3A shows the time course for inactivation of 50 nM ICE by 0 (□), 200 nM (O), 1 μM (X), or 5 μM (◇) CAP-3. Fig. 3B shows the time course for inactivation of 0.25 μM CPP32 by 0 (□) or 1 μM (X) CAP-3. Fig. 3C shows the time course for inactivation of 0.25 μM Ich-2 by 0 (□), 250 nM (O) or 1 μM (X) CAP-3. Fig. 3D shows the effect of serpin concentration on the apparent rate constant for inactivation of ICE (k_{app}) in the absence of substrate.

Fig. 4 shows the immunoprecipitation of CAP-3-caspase complexes. The complex between ICE (Fig. 4A) or Ich-2 (Fig. 4B) and CAP-3 was formed *in vitro*, precipitated with anti-CAP-3 antiserum, and separated on a 10-20% polyacrylamide gel using a Tris-tricine-SDS buffer system. The gels were immunoblotted and probed with anti-ICE p10 antibodies. Lanes contained a blocked caspase standard (S) or the immunoprecipitated complex from a reaction of blocked (B) or untreated (U) caspase incubated with (+) or without (-) CAP-3, as indicated below the gel picture.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

The present invention provides for isolated cytoplasmic antiproteinase-3 (CAP-3) proteins, polypeptides thereof, and for isolated nucleic acids encoding these proteins and polypeptides. The isolated CAP-3 nucleic acid and protein compositions can be used in a number of applications. For instance, protease degradation during protein purification is a common problem in protein chemistry. CAP-3 serpins can be used in the purification of a variety of proteins, including those of known importance in medicine and industry. As described herein, CAP-3 nucleic acid and protein compositions are also useful in the treatment of inflammatory diseases and in the treatment of diseases involving apoptosis which are

mediated by caspases. In addition, these compositions can be used in *in vitro* diagnostic procedures for these diseases.

For gene therapy using CAP-3 nucleic acid constructs containing nucleic acid encoding an intracellular mammalian serpin are introduced into a host at a dose sufficient to cause transfection of tissues and cells contacted by the nucleic acid. Expression of the nucleic acid results in treatment of the disease medicated by a caspase, such as caspases 1, 3, 4 and 8 and/or amelioration of symptoms associated with the disease. The components of the transfection vector generally include as operably linked components in the direction of transcription, a transcriptional initiation region, a DNA sequence of interest and a transcriptional termination region, wherein the transcriptional regulatory regions are functional in the host mammal's cells that are targeted for transfection. Generally, the construct does not become integrated into the host cell genome and is introduced into the host as part of a non-integrating plasmid where it is maintained primarily in an extrachromosomal or episomal form. The constructs can be either naked nucleic acid or nucleic acid associated with a lipid carrier, or other vector such as a recombinant adenovirus. By a sufficient dose is meant that which will result in a desired effect, for example, prevention, palliation, and/or cure of an animal or human disease mediated by a caspase. The desired result obtained in a multiplicity of cell types or tissues, or the modulation may be selective, for example, inducible and/or in only selected cell or tissue types, such as brain, liver, lung and/or heart. In some instances, transfection of multiple tissues and cells other than those solely at or afferent to the site of introduction of the nucleic acid constructs into the host is obtained. The route of administration typically is into a circulating bodily fluid such as blood or cerebrospinal fluid, but other routes of administration also can be used. Optionally, the construct may provide for targeting and/or expression in a particular cell type or types.

By "isolated" CAP-3 is meant to refer to CAP-3 which is in other than its native environment, and includes, for example, substantially purified CAP-3 as defined herein. More generally, isolated is meant to include CAP-3 as a heterologous component of a cell or other system. For example, CAP-3 may be expressed by a cell transfected with a DNA construct which encodes CAP-3, and then separated from the cell. Thus, in this context, the environment of isolated CAP-3 is not as it occurs in its native state, particularly when it is present in a system as an exogenous component. The predicted amino acid sequence of human

CAP-3 protein is depicted in SEQ ID NO:4. The predicted amino acid sequence of CAP-3 is 376 amino acids. The human protein has a predicted molecular weight of about 42 kDa.

Human CAP-3 protein shares some amino acid sequence identity with other members of the ovalbumin branch of the serpin superfamily of proteinase inhibitors (Sprecher *et al*, (1995) *J. Biol. Chem.* 270:29854-29861). Relatedness between these groups of proteins was calculated using the NBRF ALIGN program. Using this program, the CAP-2 (*see* U.S. Patent No. 5,712,117) and CAP-3 human proteins have 68% and 63% identity, respectively, with the human cytoplasmic antiproteinase (CAP-1) protein sequence isolated from human placenta. Morgenstern *et al*, *Biochem.* 33:3432-3441 (1994). In addition, CAP-2 shows 63% amino acid sequence identity to CAP-3. Human CAP-2 and CAP-3 also exhibit a degree of amino acid sequence identity to other human members of the ovalbumin family of cytoplasmic serpins. For instance, CAP-2 has 51% identity and CAP-3 has 49% identity with elastase inhibitor. CAP-2 and CAP-3 have 46% and 45% identity with plasminogen activator inhibitor-2, respectively. In addition, CAP-2 has 46% identity and CAP-3 has 45% identity with squamous cell carcinoma antigen.

CAP-2 and CAP-3 lack a typical N-terminal cleavable signal sequence that is present in many other members of the serpin superfamily. CAP-2 and CAP-3 also lack a C-terminal extension which is present in many serpins. CAP-2 and CAP-3 both have a serine corresponding to position 375 of α_1-proteinase inhibitor in place of a highly conserved Asn found among the serpins distantly related to the ovalbumin family. CAP-1 and CAP-2 also have potential N-glycosylation consensus motifs (N-X-T/S) starting at Asn8 and Asn78 of CAP-2 and Asn6 and Asn23 of CAP-3.

CAP-3 polypeptides typically show substantial sequence identity to the amino acid sequence of SEQ ID NO:4. As applied to these polypeptides and peptides thereof, the terms "substantial sequence identity" or "homology" or "homologous" mean that two amino acid sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap penalties, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more. "Percentage amino acid identity" or "percentage amino acid sequence identity" refers to a comparison of the amino acids of two polypeptides which, when optimally aligned, have approximately the designated percentage of the same amino acids. Preferably, residue positions which are not

identical differ by conservative amino acid substitutions. For example, the substitution of amino acids having similar chemical properties such as charge or polarity are not likely to substantially effect the properties of a protein. Examples include glutamine for asparagine or glutamic acid for aspartic acid.

The term "CAP-3 protein" refers not only to the amino acid sequences disclosed herein, but also to other proteins that are allelic or species variants of these amino acid sequences. It is also understood that these terms include nonnatural mutations introduced by deliberate mutation using recombinant technology such as single site mutation or by excising short sections of DNA encoding CAP-3 protein or by substituting new amino acids or adding new amino acids. Such minor alterations substantially maintain the immunoidentity of the original molecule and/or its biological activity. The biological properties of the altered proteins can be determined by expressing the protein in an appropriate cell line and by determining the ability of the protein to inhibit designated cysteine proteases. The biological activity of CAP-3 can be determined, for example, by its ability to inhibit proteases in the ICE family, including both serine proteases and cysteine proteases. Particular protein modifications considered minor would include substitution of amino acids of similar chemical properties, e.g., glutamic acid for aspartic acid or glutamine for asparagine.

By aligning a protein optimally with the protein of SEQ ID NO:4, and by using immunoassays as described herein to determine immunoidentity, one can readily determine the protein compositions of the invention. For example, CAP-3 protein from different mammalian species, for example, other primate species, is typically specifically immunoreactive with antibodies raised to the CAP-3 protein depicted in SEQ ID NO:4.

In other embodiments the present invention provides isolated nucleic acid molecules which encode the CAP- 3 protein. The term "isolated" as applied to nucleic acid molecules means those which are separated from their native environment, and preferably free of non-CAP-3 DNA or coding sequences with which they are naturally associated. The term "nucleic acids", as used herein, refers to either DNA or RNA. "Nucleic acid molecule" or "polynucleotide sequence" refers to a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. The nucleic acid molecules of the invention, whether RNA, cDNA, or genomic DNA, may be isolated from natural sources or may be prepared *in vitro*. The nucleic acids may be present in transformed

or transfected whole cells, in a transformed or transfected cell lysate, or in a partially purified or substantially pure form.

The nucleic acid molecules of the invention are typically identical to or show substantial sequence identity or homology (determined as described herein) to the nucleic acid molecule having a sequence shown in SEQ ID NO:3 or the complement thereof. The nucleic acid molecules include those which are equivalent to native or allelic sequences due to the degeneracy of the genetic code as well as sequences which are introduced to provide codon preference in a specific host cell. Nucleic acids encoding mammalian CAP-3 protein will typically hybridize to the nucleic acid sequences of SEQ ID NO:3 under stringent hybridization conditions. Less stringent hybridization conditions may also be selected. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt concentration is at least about 0.02 molar at pH 7 and the temperature is at least about 60°C. As other factors may significantly affect the stringency of hybridization, including, among others, base composition and size of the complementary strands, the presence of organic solvents and the extent of base mismatching, the combination of parameters is more important than the absolute measure of any one. Thus, the phrase "selectively hybridizing to" refers to a nucleic acid probe that hybridizes, duplexes or binds preferentially to a particular target DNA or RNA sequence when the target sequence is present in a preparation of total cellular DNA or RNA. "Complementary" or "target" nucleic acid sequence refers to a nucleic acid sequence which selectively hybridizes to a nucleic acid probe. For discussions of nucleic acid probe design and annealing conditions, see, for example, Sambrook *et al*, *Molecular Cloning: A Laboratory Manual* (2nd ed.), Vols. 1-3, *Cold Spring Harbor Laboratory*, (1989) or *Current Protocols in Molecular Biology*, F. Ausubel *et al*, ed., Greene Publishing and Wiley-Interscience, New York (1987), each of which is incorporated herein by reference. Techniques for manipulation of nucleic acids encoding CAP-3 protein such as subcloning nucleic acid sequences encoding polypeptides into expression vectors, labelling probes, DNA hybridization, and the like are described generally in Sambrook, *supra*.

There are various methods of isolating nucleic acid molecules encoding CAP-3 protein. For example, DNA is isolated from a genomic or cDNA library using labeled oligonucleotide probes having sequences complementary to the sequence disclosed herein (SEQ ID NO:3). Full-length probes may be used, or oligonucleotide probes also may be generated by comparison of the sequences of SEQ ID NO:3. Such probes can be used directly in hybridization assays to isolate DNA encoding CAP-3 protein. Alternatively, probes can be designed for use in amplification techniques such as PCR (Mullis, *et al*, US Patent Nos. 4,683,195 and 4,683,202, incorporated herein by reference), and DNA encoding CAP-3 protein may be isolated by using methods such as PCR. Nucleic acid probes may be DNA or RNA fragments. DNA fragments can be prepared, for example, by digesting plasmid DNA, or by use of PCR, or synthesized by either the phosphoramidite method described by Beaucage and Carruthers, (1981) *Tetrahedron Lett.* 22:1859-1862, or by the triester method according to Matteucci *et al,* (1981) *J. Am. Chem. Soc.*103:3185, both incorporated herein by reference. A double stranded fragment may then be obtained, if desired, by annealing the chemically synthesized single strands together under appropriate conditions or by synthesizing the complementary strand using DNA polymerase with an appropriate primer sequence. Where a specific sequence for a nucleic acid probe is given, it is understood that the complementary strand is also identified and included. The complementary strand will work equally well in situations where the target is a double-stranded nucleic acid.

To prepare a cDNA library, mRNA is isolated from tissue such as human placenta which expresses CAP-3 protein. cDNA is prepared from the mRNA and ligated into a recombinant vector. The vector is transfected into a recombinant host for propagation, screening and cloning. Methods for making and screening cDNA libraries are well known. See Gubler and Hoffman (1983) *Gene* 25:263-269 and Sambrook, *et al*, *supra*.

For a genomic library, the DNA is extracted from tissue and either mechanically sheared or enzymatically digested to yield fragments of about 12-20kb. The fragments are then separated by gradient centrifugation from undesired sizes and are constructed in bacteriophage lambda vectors. These vectors and phage are packaged *in vitro*, as described in *Sambrook, et al* Recombinant phage are analyzed by plaque hybridization as described in, for example, Benton and Davis (1977) *Science*, 196:180-182. Colony hybridization is carried out

as generally described in, for example, Grunstein, *et al,* (1975) *Proc. Natl. Acad. Sci. USA.*, 72:3961-3965.

DNA encoding a CAP-3 protein is identified in either cDNA or genomic libraries by its ability to hybridize with nucleic acid probes, for example on Southern blots, and these DNA regions are isolated by standard methods familiar to those of skill in the art. See Sambrook, *et al.* Various methods of amplifying target sequences, such as the polymerase chain reaction, can also be used to prepare nucleic acids encoding CAP-3 protein. PCR technology is used to amplify such nucleic acid sequences directly from mRNA, from cDNA, and from genomic libraries or cDNA libraries. The isolated sequences encoding CAP-3 protein may also be used as templates for PCR amplification.

In PCR techniques, oligonucleotide primers complementary to the two 3' borders of the DNA region to be amplified are synthesized. The polymerase chain reaction is then carried out using the two primers. See *PCR Protocols: A Guide to Methods and Applications*. Innis, M., Gelfand, D., Sninsky, J. and White, T., eds., Academic Press, San Diego (1990). Primers can be selected to amplify the entire regions encoding a full-length CAP-3 protein or to amplify smaller DNA segments as desired.

PCR can be used in a variety of protocols to isolate cDNAs encoding CAP-3 protein. In these protocols, appropriate primers and probes for amplifying DNA encoding CAP-3 protein are generated from analysis of the DNA sequences listed herein. Once such regions are PCR-amplified, they can be sequenced and oligonucleotide probes can be prepared from sequence obtained. These probes can then be used to isolate DNAs encoding CAP-3 protein, similar to the procedure used in example 2 herein. CAP-3 protein can be isolated from a variety of different tissues using this procedure.

Oligonucleotides for use as probes are chemically synthesized according to the solid phase phosphoramidite triester method first described by Beaucage and Carruthers, *Tetrahedron Lett.* (1981) 22:1859-1862 , using an automated synthesizer, e.g., as described in *Needham-VanDevanter et al,* (1984) *Nucleic Acids Res.*12:6159-6168. Purification of oligonucleotides is by either native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson and Regnier (1983) *J. Chrom.*, 255:137-149. The sequence of the synthetic oligonucleotide can be verified using the chemical degradation method of Maxam and Gilbert (1984) *Meth. Enzymol.*, 65:499-560.

Other methods known to those of skill in the art may also be used to isolate DNA molecules encoding CAP-3 protein. See Sambrook, *et al* for a description of other techniques for the isolation of DNA encoding specific protein molecules. Thus, the present invention includes nucleotide sequences that have substantial sequence identity or homology to the CAP-3 nucleotide sequences described in SEQ ID NO:3. For substantial sequence identity or homology the polynucleotide comprises a sequence that has at least 80 percent sequence identity, preferably at least 90 percent sequence identity, and more preferably at least 95 percent sequence identity. The comparison is made to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence, which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. The reference sequence may be a subset of a larger sequence, for example, as a segment of the human CAP-3 sequences described herein. Optimal alignment of sequences for aligning a comparison window may be conducted according to the local homology algorithm of Smith and Waterman (1981) *Adv. Appl. Math.* 2:482, by the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci.* (USA) 85:2444, or by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI).

Once DNA encoding CAP-3 protein or a homologous sequence is isolated and cloned, CAP-3 protein or a homologous protein can be expressed in a variety of recombinantly engineered cells. Numerous expression systems are available for expression of DNA encoding CAP-3 protein. The expression of natural or synthetic nucleic acids encoding CAP-3 protein is typically achieved by operably linking the DNA to a promoter (which is either constitutive or inducible) within an expression vector. By expression vector is meant a DNA molecule, linear or circular, that comprises a segment encoding a CAP- 3 protein or polypeptide of interest, operably linked to additional segments that provide for its transcription. Such additional segments include promoter and terminator sequences. An expression vector also may include one or more origins of replication, one or more selectable markers, an enhancer, a

polyadenylation signal, etc. Expression vectors generally are derived from plasmid or viral DNA, and can contain elements of both. The term "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, for example, transcription initiates in the promoter and proceeds through the coding segment to the terminator. *See* Sambrook *et al*, *supra*.

Expression vectors can be constructed which contain a promoter to direct transcription, a ribosome binding site, and a transcriptional terminator. Examples of regulatory regions suitable for this purpose in *E. coli* are the promoter and operator region of the *E. coli* tryptophan biosynthetic pathway as described by Yanofsky (1984) *J. Bacteriol.*, 158:1018-1024 and the leftward promoter of phage lambda (P_λ) as described by Herskowitz and Hagen, (1980) *Ann. Rev. Genet.*, 14:399-445. The inclusion of selection markers in DNA vectors transformed in *E.coli* is also useful. Examples of such markers include genes specifying resistance to ampicillin, tetracycline, or chloramphenicol. Vectors used for expressing foreign genes in bacterial hosts generally will contain a selectable marker, such as a gene for antibiotic resistance, and a promoter which functions in the host cell. Plasmids useful for transforming bacteria include pBR322 (Bolivar, *et al*, (1977) *Gene* 2:95-113), the pUC plasmids (Messing,(1983) *Meth. Enzymol.* 101:20-77, Vieira and Messing, (1982) *Gene* 19:259-268), pCQV2 (Queen, *ibid.*), and derivatives thereof. Plasmids may contain both viral and bacterial elements. Methods for the recovery of the proteins in biologically active form are discussed in U.S. Patent Nos. 4,966,963 and 4,999,422, which are incorporated herein by reference. See Sambrook, *et al* for a description of other prokaryotic expression systems.

A variety of procaryotic expression systems can be used to express CAP-3 protein. Examples include *E. coli, Bacillus, Streptomyces*, and the like. For example, CAP-3 protein can be expressed in *E. coli*. CAP-3 protein produced by prokaryotic cells may not necessarily fold properly. During purification from *E. coli*, the expressed protein may first be denatured and then renatured. This can be accomplished by solubilizing the bacterially produced proteins in a chaotropic agent such as guanidine HCl and reducing all the cysteine residues with a reducing agent such as beta-mercaptoethanol. The protein is then renatured, either by slow dialysis or by gel filtration. See U.S. Patent No. 4,511,503, incorporated herein by reference. Detection of the expressed protein is achieved by methods such as radioimmunoassay, Western blotting techniques or immunoprecipitation.

For expression in eukaryotes, host cells for use in practicing the present invention include mammalian, avian, plant, insect, and fungal cells. Fungal cells, including species of yeast (e.g., *Saccharomyces* spp., *Schizosaccharomyces* spp.) or filamentous fungi (e.g., *Aspergillus* spp., *Neurospora* spp.) may be used as host cells within the present invention. Strains of the yeast *Saccharomyces cerevisiae* can be used. As explained briefly below, CAP-3 protein can be expressed in these eukaryotic systems.

Recombinantly produced CAP-3 protein can be directed into the secretory pathway of the host cell in order to facilitate purification, by using at least one signal sequence operably linked to the DNA sequence of interest. Examples of such signals include the alpha factor signal sequence (pre-pro sequence; Kurjan and Herskowitz (1982) *Cell* 30: 933-943; Kurjan *et al*, U.S. Patent No. 4,546,082; Brake, U.S. Patent No. 4,870,008), the PHO5 signal sequence (Beck *et al*, WO 86/00637), the BAR1 secretory signal sequence (MacKay *et al*, U.S. Patent No. 4,613,572; MacKay, WO 87/002670), the SUC2 signal sequence (Carlson *et al*, (1983) *Mol. Cell. Biol.* 3: 439-447), the α-1-antitrypsin signal sequence (Kurachi *et al*, (1981) *Proc. Natl. Acad. Sci. USA* 78:6826-6830), the α-2 plasmin inhibitor signal sequence (Tone *et al*, (1987) *J. Biochem.* (Tokyo) 102:1033-1042) and the tissue plasminogen activator leader sequence (Pennica *et al (1983) Nature* 301:214-221). Alternatively, a secretory signal sequence can be synthesized according to the rules established, for example, by von Heinje (1983) *Eur. J. Biochem.* 133:17-21; (1985) *J. Mol. Biol.* 184:99-105; (1986) *Nuc. Acids Res.* 14:4683-4690.

Signal sequences may be used singly or can be combined. For example, a first signal sequence can be used singly or in combination with a sequence encoding the third domain of Barrier (described in U.S. Patent 5,037,743, incorporated by reference herein in its entirety). A DNA segment encoding the third domain of Barrier can be positioned in proper reading frame 3' of the CAP-3 DNA sequence of interest or 5' to the DNA sequence and in proper reading frame with both the signal sequence and the CAP-3 DNA sequence of interest.

Suitable yeast vectors for use in the present invention include YRp7 (Struhl *et al*, (1978) *Proc. Natl. Acad. Sci. USA* 76:1035-1039), YEp13 (Broach *et al*, (1979) *Gene* 8:121-133), POT vectors (Kawasaki *et al*, U.S. Patent No. 4,931,373, which is incorporated by reference herein), pJDB249 and pJDB219 (Beggs, (1978) *Nature* 275:104-108) and derivatives thereof. Such vectors generally include a selectable marker, which can be one of any number

of genes that exhibit a dominant phenotype for which a phenotypic assay exists to enable transformants to be selected. Preferred selectable markers are those that complement host cell auxotrophy, provide antibiotic resistance or enable a cell to utilize specific carbon sources, and include LEU2 (Broach *et al, ibid.*), URA3 (Botstein *et al*, (1979) *Gene* 8:17), HIS3 (Struhl *et al, ibid.*) or POT1 (Kawasaki *et al, ibid.*). Another suitable selectable marker is the CAT gene, which confers chloramphenicol resistance on yeast cells.

Examples of promoters for use in yeast include promoters from yeast glycolytic genes (Hitzeman *et al* (1980) *J. Biol. Chem.* 255:12073-12080; Alber and Kawasaki (1982) *J. Mol. Appl. Genet.* 1:419-434; Kawasaki, U.S. Patent No. 4,599,311) or alcohol dehydrogenase genes (Young *et al*, (1982) *Genetic Engineering of Microorganisms for Chemicals, Hollaender et al*, (eds.), p. 355, *Plenum*, New York; (1983) *Ammerer, Meth. Enzymol.* 101:192-201). The TPI1 promoter *(Kawasaki*, U.S. Patent No. 4,599,311, 1986) and the ADH2-4c promoter (Russell *et al,* (1983) *Nature* 304:652-654; and EP 284,044 also can be used. The expression units may also include a transcriptional terminator. An example of such a transcriptional terminator is the TPI1 terminator (Alber and Kawasaki, *ibid.*).

In addition to yeast, proteins of the present invention can be expressed in filamentous fungi, for example, strains of the fungi *Aspergillus* (McKnight *et al*, U.S. Patent No. 4,935,349, which is incorporated herein by reference). Examples of useful promoters include those derived from *Aspergillus nidulans* glycolytic genes, such as the ADH3 promoter (McKnight *et al*, (1985) *EMBO J.* 4: 2093-2099) and the tpiA promoter. An example of a suitable terminator is the ADH3 terminator (McKnight *et al, ibid.*). The expression units utilizing such components are cloned into vectors that are capable of insertion into the chromosomal DNA of *Aspergillus*.

Techniques for transforming fungi are well known in the literature, and have been described, for instance, by Beggs (*ibid.*), Hinnen *et al,* (1978) *Proc. Natl. Acad. Sci. USA* 75:1929-1933, Yelton *et al,* (1984) *Proc. Natl. Acad. Sci. USA* 81:1740-1747, and Russell (1983) *Nature* 301:167-169. The genotype of the host cell generally contains a genetic defect that is complemented by the selectable marker present on the expression vector. Choice of a particular host and selectable marker is well within the level of ordinary skill in the art.

In addition to fungal cells, cultured mammalian cells can be used as host cells within the present invention. Examples of cultured mammalian cells for use in the present invention

include the COS-1 (ATCC CRL 1650), BHK, and 293 (ATCC CRL 1573; Graham *et al* (1977) *J. Gen. Virol.* 6:59-72) cell lines. An example of a BHK cell line is the BHK 570 cell line (deposited with the American Type Culture Collection under accession number CRL 10314). In addition, a number of other mammalian cell lines can be used within the present invention, including rat Hep I (ATCC CRL 600), rat Hep II (ATCC CRL 1548), TCMK (ATCC CCL 139), human lung (ATCC CCL 75.1), human hepatoma (ATCC HTB-52), Hep G2 (ATCC HB 8065), mouse liver (ATCC CCL 29.1), NCTC 1469 (ATCC CCL 9.1) and DUKX cells (Urlaub and Chasin (1980) *Proc. Natl. Acad. Sci USA* 77:4216-4220).

Mammalian expression vectors for use in carrying out the present invention include a promoter capable of directing the transcription of a cloned gene or cDNA. Either viral promoters or cellular promoters can be used. Viral promoters include the immediate early cytomegalovirus (CMV) promoter (Boshart *et al*, (1985) *Cell* 41:521-530) and the SV40 promoter (Subramani *et al,* (1981) *Mol. Cell. Biol.* 1:854-864). Cellular promoters include the mouse metallothionein-1 promoter (Palmiter *et al*, U.S. Patent No. 4,579,821), a mouse Vþ promoter (Bergman *et al,* (1983) *Proc. Natl. Acad. Sci. USA* 81:7041-7045; Grant *et al*, (1987) *Nuc. Acids Res.*15:5496), a mouse VH promoter (Loh *et al*, (1983) *Cell* 33:85-93), and the major late promoter from Adenovirus 2 (Kaufman and Sharp,(1982) *Mol. Cell. Biol.* 2:1304-13199).

Promoters for tissue specific expression *in vivo* include the following. For cardiac compartment-specific expression, the alpha-myosin heavy chain promoter can be used to obtain transgene expression in adult atria and ventricles (Colbert *et al*, (1997) *Clin. Invest.* 100:1958-1968) and Masalci *et al*, (1998) 101:527-535). Expression preferentially in hepatocytes can be obtained using an albumin promoter/enhancer element (Tanaka *et al*, (1997) *Hepatology* 26:598-604). Neuron specific expression can be obtained using the neuron-specific enolase (NSE) promoter (Klein *et al*, (1998) *Exp. Neurol.* 150:183-194 and Rondi-Reig *et al*, (1997) *Neuroreport* 8:2429-2432). For specific expression in respiratory epithelial cells, the promoter from the human surfactant protein C (SP-C) gene (Xeng *et al*, (1998) *Dev. Dyn.* 211:215-227) or the regulatory elements from the human cytokeratin 18 (K18) gene (Chow *et al*, (1997) *Proc. Nat'l. Acad. Sci. U S A* 94:14695-14700) can be used.

The expression vectors also can contain a set of RNA splice sites located downstream from the promoter and upstream from the DNA sequence encoding the peptide or protein of

interest. RNA splice sites can be obtained from adenovirus and/or immunoglobulin genes. Also contained in the expression vectors is a polyadenylation signal located downstream of the coding sequence of interest. Polyadenylation signals include the early or late polyadenylation signals from SV40 (Kaufman and Sharp, *ibid.*), the polyadenylation signal from the Adenovirus 5 E1B region and the human growth hormone gene terminator (DeNoto *et al* (1981) *Nuc. Acids Res.* 9:3719-3730). The expression vectors can include a noncoding viral leader sequence, such as the Adenovirus 2 tripartite leader, located between the promoter and the RNA splice sites. Preferred vectors may also include enhancer sequences, such as the SV40 enhancer and the mouse μ enhancer (Gillies (1983) *Cell* 33:717-728). Expression vectors may also include sequences encoding the adenovirus VA RNAs.

Cloned DNA sequences can be introduced into cultured mammalian cells by, for example, calcium phosphate-mediated transfection (Wigler *et al,* (1978) *Cell* 14:725; Corsaro and Pearson, (1981) *Somatic Cell Genetics* 7:603; Graham and Van der Eb, (1973) *Virology* 52:456; which are incorporated by reference herein in their entirety). Other techniques for introducing cloned DNA sequences into mammalian cells can also be used, such as electroporation (Neumann *et al,* (1982) *EMBO J.* 1:841-845) or cationic lipid-mediated transfection (Hawley-Nelson *et al,* (1993) *Focus* 15:73-79) using, e.g., a 3:1 liposome formulation of 2,3-dioleyloxy-N-[2 (sperminecarboxyamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate and dioleoly-phosphatidylethanolamine in water Lipofectamine reagent, GIBCO-BRL). To identify cells that have integrated the cloned DNA, a selectable marker is generally introduced into the cells along with the gene or cDNA of interest. Examples of selectable markers for use in cultured mammalian cells include genes that confer resistance to drugs, such as neomycin, hygromycin, and methotrexate. The selectable marker can be an amplifiable selectable marker. A preferred amplifiable selectable marker is the DHFR gene. Selectable markers are reviewed by Thilly *(Mammalian Cell Technology*, Butterworth Publishers, Stoneham, MA, which is incorporated herein by reference). The choice of selectable markers is well within the level of ordinary skill in the art.

Selectable markers can be introduced into the cell on a separate plasmid at the same time as the gene of interest, or they may be introduced on the same plasmid. If on the same plasmid, the selectable marker and the gene of interest can be under the control of different promoters or the same promoter, the latter arrangement producing a dicistronic message.

Constructs of this type are known in the art (for example, Levinson and Simonsen, U.S. Patent No. 4,713,339). It also can be advantageous to add additional DNA, known as "carrier DNA" to the mixture which is introduced into the cells.

Transfected mammalian cells are allowed to grow for a period of time, typically 1-2 days, to begin expressing the DNA sequence(s) of interest. Drug selection is then applied to select for growth of cells that are expressing the selectable marker in a stable fashion. For cells that have been transfected with an amplifiable selectable marker the drug concentration is increased in a stepwise manner to select for increased copy number of the cloned sequences, thereby increasing expression levels.

Promoters, terminators and methods useful for introducing expression vectors encoding a CAP-3 protein of the present invention into plant, avian and insect cells have been described in the art. The use of baculoviruses, for example, as vectors for expressing heterologous DNA sequences in insect cells has been reviewed by Atkinson *et al,* (1990) *Pestic. Sci.* 28:215-224). The use of *Agrobacterium rhizogenes* as vectors for expressing genes in plant cells has been reviewed by Sinkar *et al,* (1987) *J. Biosci.* (Bangalore) 11:47-58).

Host cells containing DNA constructs of the present invention are then cultured to produce CAP-3 protein. The cells are cultured according to standard methods in a culture medium containing nutrients required for growth of the host cells. A variety of suitable media are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins, minerals and growth factors. The growth medium generally selects for cells containing the DNA construct by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker on the DNA construct or co-transfected with the DNA construct.

For gene therapy, a transgene comprising CAP-3 nucleic acid is introduced into a target cell by any of several techniques, depending at least in part on the nature of the target cell, accessability of the target cell, and the intended result of therapy. In "*ex vivo*" gene therapy, target cells are transfected *ex vivo* followed by introduction of the transformed cells into a suitable organ in the host mammal. *Ex vivo* techniques include transfection of cells *in vitro* with either naked DNA or DNA liposome conjugates, followed by introduction into a host organ ("*ex vivo*" gene therapy). The citeria for a suitable target organ or tissue include that the target organ or tissue is easily accessible, that it can be manipulated *in vitro*, that it is

susceptible to genetic modification methods and ideally, it should contain either non-replicating cells or cycling stem cells to perpetuate a genetic correction. Further, it should be possible to reimplant the genetically modified cells into the organism in a functional and stable form. Exemplary of a target organ which meets these criteria is the mammalian bone marrow. A further criterion for *ex vivo* gene therapy, if for example a retroviral vector is used, is that the cells be pre-mitotic; post-mitotic cells are refractory to infection with retroviral vectors. In some instances it is possible to transfect cells from other than the target organ or tissue using *ex vivo* gene therapy if the corrective gene product can be secreted and exert the desired effect on/in the target cell following circulation in blood or other body fluids. If only differentiated, replicating cells are infected, the newly introduced gene function will be lost as those cells mature and die, which can be advantageous for treating acute diseases and/or symptoms as the above methods generally involve integration of new genetic material into the cell genome and thus constitute permanent changes.

Liposomes can be used to introduce CAP-3 nucleic acid or CAP-3 protein into animals. The agent to be introduced is typically entrapped within the liposome, or lipid vesicle, or the agent may be bound to the outside of the vesicle. Several strategies have been devised to increase the effectiveness of liposome-mediated drug delivery by targeting liposomes to specific tissues and specific cell types. In the absence of specific targeting, liposomes are typically taken up by the liver where the transgene is expressed; generally expression is transient. Examples of liposome formulations that can be used include DOTAP™ (Boehringer-Mannheim), Tfx™-50, Transfectam®, ProFection™ (Promega, Madison, WI), and LipofectAmin™, Lipofectin®, LipofectAce™ (GibcoBRL, Gaithersburg, MD). The use of specific cationic lipids can confer specific advantages for *in vivo* delivery of complexes. For example, iv injection of nucleic acid complexed to DOTAP-containing liposomes or ethyl-phosphatidylcholine (E-PC) lipid carriers can target transgene expression primarily to the lung. Furthermore, DOTAP, as well as L-PE and cholesterol ester β-alanine (CEBA) are fully metabolized by cells, whereas DOTMA cannot be fully metabolized by cells. Therefore, DOTAP, E-PC, and L-PE, but not DOTMA, are suitable for repeated injection into mammalian hosts. Additionally, using a lipid carrier comprising a cationic lipid and a second lipid, particularly cholesterol or DOPE can maximize transgene expression *in vivo*. Also, mixing a steroid, such as cholesterol, instead of DOPE, with DOTAP, DOTMA, or DDAB,

substantially increases transgene expression *in vivo*. In solution, the lipids form vesicles that associate with the nucleic acid and facilitate its transfer into cells by fusion of the vesicles with cell membranes or by endocytosis. Liposome formulation, including those containing a cationic lipid, have been shown to be safe and well tolerated in human patients (Treat *et al*, (1990) *J. Natl. Cancer Instit.* 82:1706-1710).

In vivo expression of transgenes can also be obtained by injection of transgenes directly into a specific tissue, such as direct intratracheal, intramuscular or intraarterial injection of naked DNA or of DNA-cationic liposome complexes, or by *ex vivo* transfection of host cells, with subsequent reinfusion. The expression generally is limited to one tissue, typically the tissue that is injected (for example muscle); liver or lung where intravenous injection is used; lung where intratracheal injection is used, brain where intrathecal injection is used, and heart where injection via a catheter is used. Particular cells and tissues can be targeted, depending upon the route of administration and the site of administration. For example, a tissue which is closest to the site of injection in the direction of blood flow can be transfected in the absence of any specific targeting. Additionally, if desired, the lipid carriers may be modified to direct the complexes to particular types of cells using site-directing molecules.. Thus, antibodies or ligands for particular receptors or other cell surface proteins may be employed, with a target cell associated with a particular surface protein

Another method of introducing a transgene is using adenoviral vectors. Adenovirus-mediated gene therapy has been used successfully to transiently correct the chloride transport defect in nasal epithelia of patients with cystic fibrosis. *See* Zabner *et al*, (1993) *Cell* 75:207-216. Other examples of adenoviral vector systems are described in USPN 5,670,488 and USPN 5,631,236.

The polypeptides of the invention, including recombinantly produced CAP-3 protein produced as described above, can be purified by techniques well known to those of skill in the art. For example, recombinantly produced CAP-3 polypeptide can be directly expressed or expressed as fusion proteins. The proteins can then be purified by a combination of cell lysis (e.g., sonication) and affinity chromatography. For fusion products, subsequent digestion of the fusion protein with an appropriate proteolytic enzyme releases the desired polypeptide.

The phrase "substantially purified" when referring to CAP-2 or CAP-3 peptides or proteins of the present invention, means a composition which is essentially free of other

cellular components with which the CAP-2 or CAP-3 peptides or proteins are associated in their native environment. Purified protein is preferably in a homogeneous state although it can be in either in a dry state or in an aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. Generally, a substantially purified protein comprises more than 80% of all macromolecular species present in the preparation. Preferably, the protein is purified to represent greater than 90% of all proteins present. More preferably the protein is purified to greater than 95%, and most preferably the protein is purified to essential homogeneity, wherein other macromolecular species are not detectable by conventional techniques.

The CAP-3 peptides and proteins of the present invention can be purified to substantial purity by standard techniques well known in the art, including selective precipitation with such substances as ammonium sulfate; column chromatography; affinity methods, including immunopurification methods; and others. *See*, for instance, R. Scopes, *Protein Purification: Principles and Practice*, Springer-Verlag: New York (1982), incorporated herein by reference. For example, antibodies may be raised to the CAP-3 protein as described herein. CAP-3 protein can be extracted from tissues or cell cultures that express the protein and then immunoprecipitated. The CAP-3 protein can then be further purified by standard protein chemistry techniques as described above.

The CAP-3 protein of the present invention find use as a protease inhibitor in the purification of a wide variety of different proteins. Proteolysis is a major problem in purification of proteins. Proteolysis can occur at all stages of purification, particularly the early stages, when more contaminating proteins are present. Because of their widespread distribution, cysteine proteases often contribute to the degradation of proteins during purification. Several different cysteine protease inhibitors have been used successfully, along with other protease inhibitors, during the purification of variety of different proteins. (See, e.g., Deutscher, (1990) *Meth. Enzymol.* 182:83-89). Isolated CAP-3 protein can be used alone or in combination with a number of other protease inhibitors. CAP-3 protein is used as a cysteine protease inhibitor at a concentration of about 10 ng-100 μg/ml, typically about 1 μg/ml. In one embodiment, CAP-3 protein is covalently coupled to a solid support using conventional coupling chemistry. Suitable supports in this regard include glass beads, silica-

based resins, cellulosic resins, agarose beads, cross-linked agarose beads, polystyrene beads, cross-linked polyacrylamide resins and the like that are insoluble under the conditions in which they are to be used. These supports can be modified with reactive groups that allow attachment of proteins through amino groups, carboxyl groups, sulphydryl groups, hydroxyl groups and/or carbohydrate moieties. Examples of coupling chemistries include cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulphydryl activation, hydrazide activation, and carboxyl and amino derivatives for carbodiimide coupling chemistries. In a typical procedure, the resin-CAP complex is packed into a column, and an aqueous mixture containing a protease is applied to the column. In general, the mixture is buffered at a pH compatible with the activity optimum of the protease to maximize binding of the protease to the CAP protein. The protease is allowed to bind to the immobilized CAP protein, and other components of the mixture pass through the column. The column is washed at the pH of the loading buffer to remove additional unbound mixture components. The bound protease is eluted from the column with buffers that disrupt protein-protein interactions, such as chaotropic salts (KSCN, etc.) or high or low pH solutions. These general methods can be readily adapted for purification of proteases or removal of proteases from process streams.

The specificity of CAP-3 for inhibition of different cysteine proteases can be determined using a variety of methods. For example, isolated biologically active CAP-3 protein is added to an enzymatically active preparation of the selected cysteine protease, and enzyme activity is monitored to detect inhibition. For example, specificity and kinetics of inhibition for trypsin-like cysteine proteases can be determined as described in Morgenstern *et al,* (1994) *Biochem.* 33:3432-3441, incorporated herein by reference. As an additional example, specificity for inhibition of other proteases of interest, such as those in the ICE family, is determined by adding CAP-3 to preparations of these enzymes *in vitro* and monitoring changes in enzyme activity.

Pharmacological activity of CAP-3 and agonists or antagonists thereof can also be determined in animal model systems known to those of skill in the art. For example, CAP-3 may inhibit inflammation by inhibiting the activity of ICE or other cysteine proteases involved in the inflammation process. A number of *in vitro* and animal model systems are used for identification of compounds with anti-inflammatory activity. For example, cultured vertebrate dorsal root ganglion (DRG) neurons can be transfected with an expression vector which

encodes CAP-3 cDNA, and the inhibition of cell death or degeneration determined, as generally described in Gagliardini *et al,* (1994) *Science* 263:826-828, incorporated herein by reference. The effect of CAP-3 and antagonists or agonists thereof can be demonstrated by other methods, e.g, transfecting ICE, Ich-1L, or other gene which induces programmed cell death or cell degeneration (e.g., ced-3) into a cell which is also transfected with a gene encoding CAP-3, such as DRG, RAT-1 or HeLa cells, and exposing the cells to the potential CAP-3 agonist or antagonist compound or gene expressing said compound. See generally, Miura *et al*, (1993) *Cell* 75:653-660 and Wang *et al*, (1994) *Cell* 78:739-750, each of which is incorporated herein by reference.

The CAP-3 of the present invention also have a variety of *in vitro* diagnostic uses. For example, CAP-3 may be an endogenous inhibitor for members of the ICE family of proteases, including Ich-1. Proteases with trypsin-like specificity are involved in many physiologically important processes, and ICE and Ich-1 play important roles in inflammation and apoptosis, respectively. Because of this, determination of CAP-3 in biological samples can be useful in medicine. Levels of CAP-3 protein can be determined, for example, by means of a variety of different immunoassay procedures. Antibodies, both polyclonal and monoclonal, can be produced to CAP-3 protein and polypeptide fragments thereof according to general procedures set forth in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Pubs., N.Y. (1988), incorporated herein by reference. Antibodies raised to CAP-3 immunogen, for example, one having an amino acid sequence depicted in SEQ ID NO:4, can be selected to be specifically immunoreactive with CAP-3 proteins and not with other proteins such as CAP-1 protein and CAP-2 protein. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane, *supra*, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

Diagnostic immunoassays for CAP-3 protein in a biological sample can be performed in a variety of different formats known to those of skill in the art and described in, e.g., Harlow and Lane, *supra*; *Basic and Clinical Immunology* 7th Edition (D. Stites and A. Terr ed.) (1991); *Enzyme Immunoassay*, E.T. Maggio, ed., CRC Press, Boca Raton, Florida (1980); and "*Practice and Theory of Enzyme Immunoassays*" P. Tijssen, *Laboratory Techniques in*

Biochemistry and Molecular Biology, Elsevier Science Publishers B.V. Amsterdam (1985), each of which is incorporated herein by reference. By biological sample is meant to include body fluids and tissue specimens, that is, any sample derived from or containing cells, cell components or cell products, including, but not limited to, cell culture supernatants, cell lysates, cleared cell lysates, cell extracts, tissue extracts, blood, plasma, serum, and fractions thereof and biopsy specimen.

Expression of mRNA encoding CAP-3 protein can be detected by various procedures involving nucleic acid hybridization. A variety of nucleic acid hybridization formats are known to those skilled in the art. For example, common formats include sandwich assays and competition or displacement assays. Hybridization techniques are generally described in "*Nucleic Acid Hybridization, A Practical Approach*" Ed. Hames, B.D. and Higgins, S.J., IRL Press, 1985; Gall and Pardue, (1969), *Proc. Natl. Acad. Sci.*, U.S.A., 63:378-383; and John *et al*, (1969) *Nature*, 223:582-587, and in Sambrook, *et al*. Hybridization techniques can be also used in methods such as restriction fragment length polymorphism (RFLP) analysis to detect the presence of genetic alterations in nucleic acids encoding CAP-3 (see Sambrook, *et al*, *supra*).

The CAP-3 protein compositions, and gene, DNA and polynucleotide compositions of the present invention are useful in treatment and prevention of a variety of diseases, e.g., inflammatory diseases and diseases related to apoptosis in one or more tissues. The CAP-3 composition is used to prevent neuronal degeneration. Inhibition of βIL-1 maturation indicates the subject composition can be used to treat Alzheimer's disease, arthritis, septic shock, head injury, and other inflammatory responses.

Pharmaceutical compositions of the invention are suitable for use in a variety of drug delivery systems. Pharmaceutically acceptable carriers and formulations for use in the present invention are found in Remington's *Pharmaceutical Sciences*, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985), which is incorporated herein by reference. For a brief review of methods for drug delivery, see Langer, (1990) *Science* 249:1527-1533, which is incorporated herein by reference.

In preparing pharmaceutical compositions of the present invention, it may be desirable to modify the compositions of the present invention to alter their pharmacokinetics and biodistribution. For a general discussion of pharmacokinetics, see Remington's

Pharmaceutical Sciences, *supra*, Chapters 37-39. A number of methods for altering pharmacokinetics and biodistribution are known to one of ordinary skill in the art (See, e.g., Langer, *supra*). Examples of such methods include protection of the complexes in vesicles composed of substances such as proteins, lipids (for example, liposomes), carbohydrates, or synthetic polymers. For example, the complexes of the present invention can be incorporated into liposomes in order to enhance their pharmacokinetics and biodistribution characteristics. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka *et al*, (1980) *Ann. Rev. Biophys. Bioeng.* 9:467, U.S. Pat. Nos. 4,235,871, 4,501,728 and 4,837,028, all of which are incorporated herein by reference.

The CAP-3 protein of the present invention can be used in pharmaceutical compositions that are useful for administration to mammals, including humans. The pharmaceutical compositions of the invention are intended for parenteral, topical, oral or local administration. For example, the pharmaceutical compositions can be administered parenterally, e.g., intravenously, subcutaneously, intradermally, or intramuscularly. The invention provides compositions that comprise a solution of the agents described above dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of pharmaceutically acceptable aqueous carriers may be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid and the like. These compositions can be sterilized by conventional, well known sterilization techniques, or can be sterile filtered. The resulting aqueous solutions can be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain as pharmaceutically acceptable carriers, substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

For solid compositions, conventional nontoxic pharmaceutically acceptable carriers can be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the

normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient and more preferably at a concentration of 25%-75%.

For aerosol administration, the pharmaceutical compositions containing the CAP-3 protein or peptide fragments thereof are preferably supplied in finely divided form along with a surfactant and propellant as pharmaceutically acceptable carriers. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides, can be employed. A carrier also can be included, as desired, as with, for example, lecithin for intranasal delivery.

The pharmaceutical compositions of the invention can be administered in a variety of unit dosage forms depending upon the method of administration. For example, unit dosage forms suitable for oral administration include powder, tablets, pills, and capsules. The effective amount of the CAP-3 protein in a pharmaceutical composition depends on, for example, the protein composition, the manner of administration, the weight and general state of health of the patient, the severity of the disease being treated and the judgment of the prescribing physician. Dosages, formulations and administration schedules can vary in these patients as compared to normal individuals. In general, dosages range from about 100 μg to about 500 mg or more, with dosages of from about 250 μg to about 50 mg being more commonly used. It must be kept in mind that the materials of the present invention may generally be employed in serious disease or injury states, and in such cases it is possible and may be felt desirable by the treating physician to administer substantial excesses of these CAP-3 compositions.

The mammalian host may be any mammal having symptoms of a disease mediated by a capsase, including an infectious disease which is amenable to therapy with an intracelluar serpin, particularly a mammalian intracellular serpion. Thus, the subject application finds use in domestic animals, feed stock, such as bovine, ovine, and porcine, as well as primates, particularly humans. The mammalian host may be pregnant, and the intended recipient of the therapy may be either the gravid female or the fetus or both.

In the gene therapy method of the invention, transfection *in vivo* is obtained by introducing a therapeutic transcription or expression vector into the mammalian host, either as naked DNA, complexed to lipid carriers, particularly cationic lipid carriers, or inserted into a viral vector, for example a recombinant adenovirus. The constructs may provide for integration into the host cell genome for stable maintenance of the transgene or preferably for episomal expression of the transgene. The introduction into the mammalian host may be by any of several routes, including intravenous or intraperitoneal injection, intratracheally, intrathecally, parenterally, intraarticularly, intranasally, intramuscularly, topically, transdermally, application to any mucous membrane surface, corneal installation, etc. Of particular interest is the introduction of a therapeutic expression vector into a circulating bodily fluid or into a body orifice or cavity, such as heart, lung, colon, vagina, and the like. Thus, intravenous administration and intrathecal administration are of particular interest since the vector may be widely disseminated following such routes of administration, and aerosol administration finds use with introduction into a body orifice or cavity. Any physiologically acceptable medium may be employed for administering the DNA or lipid carriers, such as deionized water, saline, phosphate-buffered saline, 5% dextrose in water, and the like as described above for the CAP-3 protein, depending upon the route of administration. Other components may be included in the formulation such as buffers, stabilizers, biocides, etc. These components have found extensive exemplification in the literature and need not be described in particular here. Any diluent or components of diluents that would cause aggregation of the complexes should be avoided, including high salt, chelating agents, and the like.

The amount of therapeutic vector used will be an amount sufficient to provide for a therapeutic level of expression in a diseased target tissue or for adequate dissemination to a variety of tissues after entry into the bloodstream and to provide for a therapeutic level of expression in diseased target tissues. A therapeutic level of expression is a sufficient amount of expression to prevent, treat, or palliate a disease or infection of the host mammal or the symptoms of a disease or infection. In addition, the dose of the nucleic acid vector used must be sufficient to produce a desired level of transgene expression in the target diseased tissue or tissues *in vivo*. Other DNA sequences, such as adenovirus VA genes can be included in the administration medium and be co-transfected with the gene of interest. The presence of genes

coding for the adenovirus VA gene product may significantly enhance the translation of mRNA transcribed from the plasmid if this is desired.

A number of factors can affect the amount of expression in transfected tissue and thus can be used to modify the level of expression to fit a particular purpose. Where a high level of expression is desired, all factors can be optimized, where less expression is desired, one or more parameters can be altered so that the desired level of expression is attained. For example, if high expression would exceed the therapeutic window, then less than optimum conditions can be used.

The level and tissues of expression of the recombinant gene may be determined at the mRNA level as described above, and/or at the level of polypeptide or protein. Gene product may be quantitated by measuring its biological activity in tissues. For example, CAP-3 protein activity can be measured by immunoassay as described above, by biological assay such as inhibition of ICE, or by identifying the gene product in transfected cells by immunostaining techniques such as probing with an antibody which specifically recognizes the gene product or a reporter gene product present in the expression cassette. Alternatively, potential therapeutic effects of the gene product can be measured, for example, by determining the effects of gene expression on survival of lethally irradiated animals in which the GM-CSF transgene is expressed. Production of significant amounts of a transgene product will substantially prolong the survival of these mice.

Typically, the therapeutic cassette is not integrated into the host cell genome. If necessary, the treatment can be repeated on an *ad hoc* basis depending upon the results achieved. If the treatment is repeated, the mammalian host can be monitored to ensure that there is no adverse immune or other response to the treatment. As an example, in a clinical setting where it is desired to treat a particular disease state, both the biological efficacy of the treatment modality as well as the clinical efficacy need to be evaluated, if possible. For example, in the treatment of acute liver failure consequent to inhalation, ingestion or parenteral administration of an industrial toxin such as carbon tetrachloride, there is a loss of liver function which manifests itself by jaundice and hepatomegaly and elevation of serum transaminase levels. The biological efficacy of gene therapy therefore can be evaluated, for example, by observation of the amount of jaundice and by measuring the serum transaminase levels prior to treatment and following transfection of liver cells. The clinical efficacy,

whether treatment of the underlying defect is effective in changing the course of disease, can be more difficult to measure. While the evaluation of the biological efficacy goes a long way as a surrogate endpoint for the clinical efficacy, it is not definitive. Thus, measuring a clinical endpoint which can give an indication of liver function after, for example, a six-month period of time, may give an indication of the clinical efficacy of the treatment regimen. Similarly, one skilled in the art can evaluate the biological and clinical efficacy of a particular gene therapy protocol for other diseases mediated by caspases and susceptible to treatment using a CAP-3 transgene.

The subject compositions can be provided for use in one or more procedures. Kits usually will include the therapeutic CAP-3 DNA either as naked DNA, as recombinant adenovirus, or complexed to lipid carriers. Additionally, lipid carriers may be provided in a separate container for complexing with the provided DNA. The therapeutic DNA may be present as concentrates which may be further diluted prior to use or they may be provided at the concentration of use, where the vials may include one or more dosages. Conveniently, single dosages may be provided in sterile vials so that the physician or veterinarian may employ the vials directly, where the vials will have the desired amount and concentration of agents. When the vials contain the formulation for direct use, usually there will be no need for other reagents for use with the method.

The invention finds use in *in vivo* prevention, treatment and/or palliation of a number of diseases that are mediated by caspases, particularly diseases characterized by and/or due to inflammation and/or apoptosis. These diseases include viral diseases which induce apoptosis through a caspase-dependent, serpin-sensitive pathway, for example, infections due to, for example, Sindbis virus, Semliki Forest virus (*Togarviridae* [Arboviruses]), Hantaviruses (*Bunyaviridae*), Lassa virus (*Arenaviridae*), Parvoviruses (*Parvoviridae*), Ebola virus (*Filoviridae*), and Enteroviruses (*Picornaviridae*); neurodegenerative disorders, such as Alzheimers' disease, Parkinson's disease, ALS, hypoxia-ischemia, and trauma; modification of vascular responses in xenotransplantation; endothelial cell hypoxic and reperfusion injury, and liver diseases such as those that result in acute liver failure as seen following exposure to toxins or as a result of cirrosis or hepatitis B infection. Other diseases include cancers, where the therapeutic transgene is an antisense molecule of the CAP-3 gene.

The following examples are offered by way of illustration of the present invention, not limitation.

EXAMPLES

Example 1

Isolation of cDNA molecules encoding human CAP-3 protein.

A. Generation of a nucleic acid probe for screening a human cDNA library.

To isolate cDNA molecules encoding CAP-1 and CAP-2 proteins, a human placenta λgt11 cDNA library was screened using an antisense 209 base pair PCR-generated ^{32}P-labeled probe corresponding to codons encoding residues 67-149 of the CAP-1 protein. (*See* Morgenstern, *et al* (1994) *supra*.) This probe was generated in a series of PCR reactions, as described below.

In the first PCR reaction, a human placenta cDNA library (Cat. # HL 1075b, ClonTech, Palo Alto, CA) was amplified using oligonucleotide ZC6657 (SEQ ID NO: 5) and oligonucleotide ZC6658 (SEQ ID NO:6). These oligonucleotides are degenerate primers based on a peptide sequence isolated from the CAP-1 protein. (*See* Morgenstern, *et al* (1994) *supra*.) The PCR reaction was generated using Ampliwax™ (Roche Molecular Systems, Branchburg, NJ) and a "hot start" technique that prevents false priming by adding the enzyme to the PCR reaction at an elevated temperature. More specifically, PCR was performed by mixing 21 μl H2O, 8 μl dNTP (2.5 mM each dNTP), 8 μl of 20 pM/μl oligonucleotide ZC6657, 8 μl of 20 pM/μl oligonucleotide ZC6658, and 5 μl of 10x GeneAmp® PCR buffer. Ampliwax™ was added, and the reaction mixture was heated at 80°C for 5 minutes and then at 35°C for 2 minutes. After the wax hardened on top of the reaction mixture, 2 μl of the human placenta cDNA library was diluted into 42 μl of water and boiled for 5-10 minutes. The diluted cDNA library was added above the cooled wax with 1 μl AmpliTaq® (5 units/μl; Roche) and 5 μl of 10x GeneAmpþ PCR buffer (Roche). The reaction mixture was incubated for 30 cycles of the following temperatures: 95°C for 30 seconds; 48øC for 30 seconds; and 72°C for 1 minute. This was followed by a 7 minute incubation at 72°C.

The product of the first PCR reaction was then used as a template in a second PCR reaction to generate a product of approximately 270 bp. A reaction mixture was prepared by

combining 21 μl H_2O, 8 μl dNTPs (2.5 mM each), 8 μl of the ZC6657 oligonucleotide (20 pM/μl, 8 μl of the ZC6658 oligonucleotide (20 pM/μl), and 5 μl of 10x GeneAmp® PCR buffer. Ampliwax™ was added, and the reaction mixture was heated at 80°C for 5 minutes, then at 35°C for 2 minutes. After the wax hardened, 43 μl H_2O, 1 μl AmpliTaq® (5 units/μl), 5 μl 10x GeneAmp® PCR buffer, and 1 μl template DNA were added above the cooled wax. The reaction mixture was then incubated for 30 cycles at the following temperatures: 95°C for 30 seconds; 48°C for 30 seconds; and 72°C for 1 minute. This was followed by a 7 minute incubation at 72°C. The final PCR product was digested with *Eco*RI and ligated into the *Eco*RI digested vector ZEM 228CC, described below. The resulting construct was designated "clone 10 CAP-Zem228CC".

The vector Zem228CC was prepared from plasmid Zem228, a pUC18-based expression vector containing a unique *Bam*HI site for insertion of cloned DNA between the mouse metallothionein-1 promoter and SV40 transcription terminator and an expression unit containing the SV40 early promoter, neomycin resistance gene, and SV40 terminator. Zem228 was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD on September 28, 1993 as an *E. coli* HB101 transformant. It has been assigned Accession Number 69446. Plasmid Zem228 was modified to delete the two *Eco*RI sites by partial digestion with *Eco*RI, blunting with DNA polymerase I (Klenow fragment) in the presence of dNTPs, and re-ligation. Digestion of the resulting plasmid with *Bam* HI followed by ligation of the linearized plasmid with *Bam*HI-*Eco*RI adapters resulted in a unique *Eco*RI cloning site. The resultant plasmid was designated Zem228R. The *Sst*I site between the SV40 promoter and the mouse metallothionein-1 promoter was destroyed by linearizing Zem228R with *Sst*I, blunting the adhesive ends with T4 DNA polymerase in the presence of dNTPs and religating the linearized, blunt-ended fragment. A plasmid in which the *Sst*I site was destroyed was designed Zem228Ra.

To facilitate directional insertion of cDNA fragments into the vector, an adapter was synthesized which contained a 5' *Eco*RI adhesive end, an internal *Sst*I site and a 3' *Eco*RI adhesive end that does not regenerate an *Eco*RI site upon ligation with an *Eco*RI adhesive end. Plasmid Zem228Ra was linearized by digestion with *Eco*RI, and the linearized plasmid was treated with calf alkaline phosphatase to prevent recircularization. The linearized plasmid was

ligated with kinased oligonucleotides ZC3169 and ZC3168 (*see* Table). A plasmid containing inserted adapter was designated Zem228C.

To improve the ability to achieve *Eco*RI + *Sst*I cleavage of the Zem228C vector, an oligonucleotide adapter was synthesized that contained an internal *Eco*RI site flanked by *Eco*RI adhesive ends that do not regenerate *Eco*RI sites upon ligation with *Eco*RI adhesive ends. Oligonucleotides ZC1773 and ZC1774 (*see* Table) were kinased and annealed to form the adapter. Plasmid Zem228C was linearized by digestion with *Eco*RI, and the linearized vector and kinased adapter were ligated. A plasmid containing the adapter was confirmed and sequenced. Sequence analysis revealed that the plasmid contained a 30 bp DNA insert between the new *Eco*RI site and the downstream *Sst*I site. Since an *Eco*RI + *Sst*I cleavage of the vector prior to the insertion of a cDNA sequence removes the additional DNA sequence, the inserted DNA was not removed. The plasmid was designated Zem228CC.

Table

Plasmid	Oligonucleotide Sequence
ZC1773:	AATTAGGGAG ACCGGAATTC TGTGCTCTGT CAA (SEQ ID NO:13)
ZC1774:	AATTTTGACA GAGCACAGAA TTCCGGTCTC CTT (SEQ ID NO:14)
ZC3168:	AATTGAGCTC G (SEQ ID NO:15)
ZC3169:	AATTCGAGCT C (SEQ ID NO:16)

The plasmid clone 10 CAP-Zem 228CC was used as a template to generate a probe for screening a human placenta cDNA library for nucleic acid sequences structurally related to CAP-1. A PCR reaction was carried out using the plasmid template and the oligonucleotide primers ZC6770 (SEQ ID NO:7) and ZC6771 (SEQ ID NO:8). PCR was performed in a 50 μl reaction volume with approximately 10 ng plasmid DNA, 40 pmoles of oligonucleotide ZC6770, 40 pmoles of oligonucleotide ZC6771, 5 μl of 10x GeneAmpþ PCR buffer, 5 μl dNPS (2.5 mM each dNTP), and 0.25 μl AmpliTaq® DNA polymerase (5 Units/μl). The reaction mixture was incubated for 35 cycles at the following temperatures: 94°C for 1 minute; 55°C for 1 minute; and 72°C for 1.5 minutes. This was followed by a 7 minute incubation at 72°C. The 210 bp product was labeled with ^{32}P using a commercially available kit (Multi-prime kit, Amersham Corporation, Arlington Heights, Illinois, USA).

B. Library screening and isolation and characterization of cDNA clones encoding human CAP-2 and CAP-3 proteins.

33,000 phage from the human placenta cDNA library were plated on each of 23 plates to obtain 760,000 independent phage. Filter lifts were made from each plate using ICN BioTrans nylon membranes. Filters were prehybridized in 5 x SSPE (20X SSPE is 175.3 g NaCl, 27.6 g NaH2PO4.H2O, 7.4 g EDTA, NaOH added to pH7.4 and water to 1 liter), 5 x Denhardt's, 0.5% SDS, 100 μl/ml salmon sperm DNA at 65°C for 6 hours. Filters were then hybridized in the above mixture with 1 x 106 cpm/ml of labeled CAP probe at 65°C overnight. The filters were washed 3 times for 40 minutes each at room temperature in 0.2 x SSC, 0.1% SDS, followed by 1 wash for 40 minutes at 65°C. Filters were exposed to X-ray film overnight. Two types of positive spots were observed: those which were extremely intense and corresponded to CAP cDNA, and those that gave much weaker signals.

For two of the weak signals, the corresponding phage were plaque-purified by additional rounds of hybridization and isolation using nitrocellulose filters. In some cases, filters were washed prior to hybridization in 5 x SSC, 0.1% SDS at 65°C for 1 hour; hybridization was carried out in 5 x SSC at 56°C overnight; and filters were washed at 58°C in 2 x SSC, 0.1% SDS. Two plaques were purified by this procedure. The two purified plaques were designated H2-2-11 and H3-1-11.

cDNA inserts were isolated from each purified plaque by PCR, using phage as a template and amplifying with oligonucleotide primers ZC2682 (SEQ ID NO:9) and ZC2683 (SEQ ID NO:10), which anneal 5' and 3' to the *Eco*RI cloning site of λgt11. A 50 μl PCR reaction volume was used with 1 μl eluted phage, 40 pmoles of oligonucleotide ZC2682, 40 pmoles of oligonucleotide ZC2683, 5 μl 10x PFU buffer (Stratagene Cloning Systems, Stratagene, San Diego, California, USA), 5 μl dNTPs (2.5 mM each dNTP; Roche), and 1 μl PFU polymerase, 2.5 Units/μl (Stratagene, San Diego, California, USA). The reaction mixture was incubated for 35 cycles at the following temperatures: 94°C for 1 minute; 60°C for 1 minute; and 72°C for 2 minutes. This was followed by a 7 minute incubation at 72°C. The resulting PCR products were designated H2-2-11 and H3-1-11.

The H2-2-11 and H3-1-11 PCR products were digested with *Eco*RI and individually ligated into the *Eco*RI site of pUC19. The H2-2-11 sequence was isolated as a 1.4 kb fragment. H3-1-11 contained an internal *Eco*RI site in the cDNA, resulting in a 1.1 kb

fragment and a 0.3 kb fragment upon digestion with *Eco*RI. Both cDNAs were sequenced and determined to be related to CAP-1 and other intracellular members of the serpin family of protease inhibitors. The nucleotide sequences and the deduced amino acid sequences of the inserts in clones H3-1-11 and H2-2-11 are shown in SEQ ID NOS:1-4. For both cDNAs, the active site region was sequenced from 2 independent PCR products to check for any PCR-generated errors.

The 5'-regions of both cDNAs contain a Kozak consensus sequence between nucleotide bases 110-119 of H2-2-11 (SEQ ID NO:3) and 88-97 of H3-1-11 (SEQ ID NO:1) that includes an in-frame initiation codon. A second Kozak sequence also exists 117 nucleotide bases downstream of the first initiation codon and includes the codon for Met41 of both proteins. The 3'-untranslated region of the H3-1-11 cDNA contains an AATAAA consensus sequence located 99 nucleotide bases downstream of the termination codon for nascent mRNA cleavage and polyadenylation. However, a polyadenylation consensus sequence was not found in the 3'-untranslated region of the H2-2-11 cDNA after sequencing 151 nucleotides downstream from the translational termination codon.

Alignment of the deduced primary structure of CAP-2 and CAP-3 with the amino acid sequences of CAP-1 and other human members of the ovalbumin serpin family identified the putative reactive center P_1P_1' residues of CAP-2 as Arg341- Cys342, respectively, which are identical to the CAP-1 reactive center residues. However, the regions flanking the P_1P_1' residues in CAP-1 and CAP-2 are highly divergent. The P_2P_6 residues of CAP-1 and CAP-2 show no identity while Arg346 in the P_3' position was conserved in both serpins. Residues in the vicinity of P_1 have been shown previously to influence both proteinase target specificity and the inhibitory potency of several serpins. Carrell *et al,* (1991) *Nature* 353:576-578. Thus, this indicates that CAP-2 interacts with the active sites of distinct cognate proteinases that have trypsin-like substrate specificity. In contrast, alignment of the CAP-3 amino acid sequence identified the putative P_1P_1' residues at Glu341-Cys342, respectively.

The identification of an acidic P_1 residue in the CAP-3 reactive center is unique in the mammalian serpin superfamily. The only other serpin identified with an acidic P_1 residue in the reactive center is encoded by the crmA gene of the cowpox virus, which has been previously demonstrated to have a reactive center containing an Asp-Cys in the P_1P_1' positions, respectively. Furthermore, the crmA protein shows the greatest degree of amino acid sequence

identity to the mammalian intracellular serpins of the ovalbumin family. By employing the NBRF program ALIGN, the crmA protein was found to share about 37% identity with CAP-2 and CAP-3. The CAP-3 reactive center domain shares about 54% of structurally conserved residues found in the reactive center domain of the crmA protein, including a conserved Asp to Glu switch in the P_1-specificity site. Moreover, the cytoplasmic antiproteinases have a unique Cys-residue conserved in the P_2' position and found only in the corresponding position of the crmA serpin reactive center.

The crmA protein functions as a specific inhibitor of ICE which represents a prototype of a larger family of ICE-like homologs. The ICE family of cysteine proteinases have been linked to both the negative and positive regulation of apoptosis. A human homolog of ICE has been identified and designated as Ich-1. In contrast to ICE, Ich-1-mediated effects on apoptosis of Rat-1 cells is only partially blocked by either microinjected or coexpressed crmA protein. These findings suggest that Ich-1 and the crmA serpin interact weakly and further suggests that Ich-1 and ICE have distinct but overlapping substrate specificities.

Serp2-2, the cDNA encoding CAP-3 (H2-2-11), inserted into pUC19 as an *Eco*RI fragment; Serp3-1a (H3-1-11), the 5' *Eco*RI fragment of the CAP-2 clone inserted into pUC19; and Serp3-1b (H3-1-11), the 3' *Eco*RI fragment of the CAP-2 clone inserted into pUC19, were deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD on October 6, 1994 as E. coli DH10b transformants and assigned Accession Numbers 69699, 69700, and 69701, respectively.

Example 2

Northern blot analysis of human tissues for expression of CAP-2 and CAP-3 mRNA

To identify transcripts that encode CAP-2 and CAP-3 and determine to their human tissue distribution, radiolabeled oligonucleotide probes corresponding to the reactive centers of these serpins were used to probe immobilized poly(A)+ mRNA by Northern analysis. A blot of poly(A)-containing mRNA from human tissues used a multiple tissue blot from CLONTECH Laboratories, Palo Alto, CA, USA. The multiple tissue blot was probed with a 39-mer oligonucleotide corresponding to the reactive centers of the CAP homologs. The oligonucleotide used for CAP-2 has the following sequence:

5'-CTCCATTCTGCTGCACCGGGAATTCCTGACCACAGCAGT-3' (SEQ ID NO: 11). The oligonucleotide used for CAP-3 has the following sequence: 5'-AGATTCCATGCAGCACTCTGCAACTACAAAGCAGCTCGA-3' (SEQ ID NO:12). The oligonucleotide probes were 5'-labeled with [$^{32}P_{\gamma}$]ATP (Dupont/NEN, Boston, Massachusetts, USA) using T4 polynucleotide kinase (Promega Corporation, Madison, Wisconsin, USA) to yield a specific activity of about 1-2 X 10^8 cpm/æg. Hybridization was performed at 55°C in 5X SSPE, 2X Denhardt's,0.5 SDS, 100 μg/ml salmon sperm DNA. The blots were washed at 57°C in 2X SSC, 0.1% SDS and exposed to autoradiography.

The results indicated that two mRNA species of 3.4 kbp and 4.4 kbp were detected with the CAP-3 reactive center antisense probe. Both mRNA species encoding the CAP-3 reactive center were detected at the highest levels in placenta and lung and to a much lesser degree in all other tissues examined. In addition, two minor mRNA species of approximately 7.5-8.0 kbp were also detected in these tissues (placenta and lung). The hybridization of the Northern blots was of sufficient stringency to preclude hybridization of inexact nucleotide matches.

Example 3

Inhibition of Caspases by CAP-3.

Materials

Ni-NTA-agarose resin was from QIAgen (Chatsworth, CA). Ac-Tyr-Val-Ala-Asp-*p*-nitroanilide (AcYVAD-pNA) and carbobenzyloxyglycine *p*-nitrophenyl ester were from Bachem (King of Prussia, PA). Ac-Asp-Glu-Val-Asp-*p*-nitroanilide (AcDEVD-pNA) was from California Peptide Research (Napa, CA). PBS was from Digene Diagnostics (Beltsville, MD). Cathepsin B, its substrate carbobenzyloxy-Arg-Arg-(7-aminomethyl)coumarin, and papain were obtained from Sigma (St. Louis, MO). Biotin-Tyr-Val-Ala-Asp-acyloxymethylketone (ICE Inhibitor III, Biotin conjugate) as purchased from Calbiochem (La Jolla, CA). Mouse anti-CPP32 monoclonal antibody was obtained from Transduction Laboratories (Lexington, KY). Rabbit polyclonal antibodies against ICE p10 and ICE p20 were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anit-Ich-2 IgG was a generous gift from D. Banach (BASF Bioresearch Corporation, Worcester, MA). Horseradish peroxidase-labeled donkey anti-rabbit IgG was from Amersham (Arlington Heights, IL). Goat

anti-rabbit IgG and horseradish peroxidase-labeled goat anti-mouse IgG were fromOncogen Sciences (Cambridge, MA).

Preparation of caspases.

Recombinant ICE was prepared from *E. coli* BL21 (DE3)/pLysS bearing a plasmid containing the full length cDNA for ICE by affinity chromatography on aldehyde inhibitor-linked resin as described (7). This ICE contained glutathione covalently bound to the active site thiol to stabilize the enzyme for storage. Dithiothreitol was included in assays and incubations to remove this ligand. His-tagged CPP32 and Ich-2 were purified on Ni-NTA-agarose as described in Kamens *et al,* (1995) *J. Biol. Chem* 270:15250-15256.

Preparation of CAP-3.

Recombinant CAP-3 was purified from transformed yeast lysates by heparin-agarose and ion exchange chromatography as described (35). The Mono-Q-purified CAP-3 was dialyzed at 4°C against 25 mM sodium citrate, 1 mM dithiothreitol, pH 5.1, and subsequently applied to a Mono S FPLC HR5/5 column equilibrated at room temperature with the above buffer. CAP-3 was eluted from the column in a linear gradient of NaCl (0 - 1 M) in equilibrating buffer. Fractions from the Mono S column were analyzed by SDS-PAGE, and fractions containing purified CAP-3 were pooled and stored at -80°C.

Anti-CAP-3 Antibodies

Antibodies against pure recombinant CAP-3 were generated in rabbits (Dunbar *et al,* (1990) *Methods Enzymol.* 182:663-670), and the IgG fraction was purified by protein A-Sepharose column chromatography.

Kinetic Characterization of Proteinase Inhibition by CAP-3

The hydrolysis of tetrapeptide *p*-nitroanilides by caspases was measured using a Cary 4 UV spectrophotometer (Varian Instruments, Palo Alto, CA). The substrate was AcYVAD-pNA for ICE and Ich-2 and AcDevD-pNA for CPP32. The reactions were carried out in 1 mL HE (100 mM Hepes, 20% glycerol, 0.5 mM EDTA, pH 7.4) containing 0.5 mg/mL BSA (bovine serum albumin), substrate, and enzyme (1.535 nM ICE, 0.15 nM CPP32, or 50 nM Ich-2). Dithiothreitol (5 mM) was added to ICE and Ich-2 assays to stabilize the enzyme. A control experiment showed no effect of dithiothreitol on the inhibition of CPP32 by CAP-3, and dithiothreitol destabilized this enzyme, so it was omitted from reactions containing CPP32. The release of *p*-nitroaniline at 30°C was followed at 380 nm for 10 min, and an aliquot of

CAP-3 was added. The reaction was followed for an additional 140 min, and the amount of released *p*-nitroaniline was calculated using ε 380nm = 14600 M^{-1} cm^{-1}, which was determined using authentic *p*-nitroaniline in the same buffer.

To measure the time course of inactivation in the absence of substrate, ICE (50 nM) and CAP-3 (0 to 5 μM) were incubated at 30°C in a 90 μL reaction containing HE, 0.5 mg/mL BSA and 5 mM DTT. At different times, a 15 μL aliquot of the reaction was diluted ten-fold into an assay mixture containing 5mM dithiothreitol, 0.5/mg/mL BSA, and 200 μM AcYVAD-pNA (final concentrations) in HGE which had been preequilibrated at 30°C. The release of *p*-nitroaniline was followed spectroscopically for 10 min at 380 nm using the Cary 4 spectrometer.

To measure the inactivation rate of Ich-2 and CPP32 in the absence of substrate, a slightly different experiment was performed using a 96-well plate reader (Molecular Devices, Sunnyvale, CA). Solutions (200 μL final volume) containing HE plus 0.5 mg/mL BSA, 5 mM dithiothreitol, and 0 to 2 μM CAP-3 (final concentrations) were incubated at 30°C in one column of wells. Proteinase was added simultaneously to all these wells with a multichannel pipettor to a final concentration of 0.25 μM to start the reaction, and 20 μL aliquots were periodically transferred to wells containing 80 μL HGE plus 0.5 mg/mL BSA, 5 mM dithiothreitol and the appropriate *p*-nitroanilide substrate. With CPP32, the substrate was 200 μM AcDEVD-pNA; with Ich-2, 1mM AcYVAD-pNA. The release of product was followed for 5 min by UV absorbance at 405 nm.

Inhibition of cathepsin B was measured by preincubating 0.5 μM enzyme with 1 μM CAP-3 in HE containing 2 mM dithiothreitol. The cathepsin B activity was assayed using the fluorescent substrate carbobenzyloxy-Arg-Arg-(7-aminomethyl)coumarin as described in Barrett *et al* (1981) *Methods Enzymol.* 80:535-561.

Inhibition of papain by CAP-3 was measured using the 96-well plate assay as described above for ICE. Papain was activated at 4°C for 20 min in 50 mM potassium phosphate, 0.5 mM EDTA, 20 mM cysteine, pH 7.0, and the cysteine was removed using a BioGel P-6 spin column (BioRad Laboratories, Hercules, CA) pre-equilibrated with 50 mM potassium phosphate, 0.5 mM EDTA, pH 7.0. Activated papain was added to the preincubation solution containing 50 mM potassium phosphate, 0.5 mM EDTA, pH 7.0, and 0 or 1 μM CAP-3 (final concentration) to give a final concentration of 0.5 μM to start the reaction. Aliquots of the

preincubation mixture were added periodically to an assay mixture containing 625 μM carbobenzyloxyglycine *p*-nitrophenyl ester in 50 mM potassium phosphate, 0.5 mM EDTA, pH 7.0, and release of *p*-nitrophenol was followed at 405 nm (Klein *et al,* (1969) *J. Biol. Chem.* 244:5928-5935).

SDS-PAGE of ICE-CAP-3 Complex.

A solution of 670 nM ICE and 1.33 μM CAP-3 in 50 μL 100 mM Hepes, 10% sucrose, 0.1% CHAPS, 5 mM dithiothreitol, pH 7.4, was reacted at 30°C. At various times, a 10 μL aliquot was quenched by added 12 μL 2X gel loading buffer (Integrated Separation Systems, Hyde Park, MA) containing 10% β-mercaptoethanol and heating at 100°C for 10 min. Duplicate 10-20% gradient polyacrylamide gels (Novex Experimental Technologies, San Diego, CA) were loaded with 10 μL samples and run in a Tris-tricine buffer system according to the manufacturer's instructions. One gel was silver stained using the Novex kit, and the other was blotted onto nitrocellulose in Novex blotting buffer at 215 mA for 1 hr. The nitrocellulose was blocked with 2% BSA, 2% non-fat dry milk, 0.3% Tween-20 in PBS and analyzed by Western blotting using the ECL kit (Amersham). Five microliters of the primary antibody, anti-CAP-3 (4.6 mg/mL), was added directly to the blocking solution (25 mL). The secondary antibody, horseradish peroxidase-coupled donkey anti-rabbit IgG, was used at 1/5000 dilution. After exposure, the blot was stripped according to the ECL kit instructions and reprobed with anti-ICE antibodies in an analogous manner.

Immunoprecipitation of ICE-CAP-3 Complex.

Solutions of ICE (1.5 μM) and Ich-2 (6 μM) were treated with a ten-fold excess of the ICE inhibitor biotin-Tyr-Val-Ala-Asp-acyloxymethylketone in HE buffer for 1 hr at 30°C to covalently block the active sites (Thornberry *et al* (1994) *Biochemistry* 33:3934-3940). CPP32, blocked or untreated ICE, blocked or untreated Ich-2, CAP-3, or a mixture of caspase and CAP-3 (1.0 μM each protein) was incubated in 20 μL HE with 0.5 mg/mL BSA, 5 mM dithiothreitol at 30°C for 1 hr, and 500 μL of ice-cold TBS-T (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, pH 7.4) containing 10 mg/mL BSA was added to stop the reaction. Anti-CAP-3 antibodies (2 μL, 4.6 mg/mL) were added to each reaction, and the solutions were mixed at 4°C for 1 hr. Protein A-agarose (20 μL) was added, and the suspensions were mixed for an additional hour at 4°C and centrifuged. The pellets were washed twice with TBS-T + BSA and once with TBS-T. The pellets were suspended in

approximately 20 μL of the last wash, and 20 μL 2 X gel loading buffer containing 10% β-mercaptoethanol was added. The samples were heated to 100°C for 10 min, and 20 μL of each sample was run on a 14% polyacrylamide gel using a Tris-glycine buffer or on a 10-20% polyacrylamide gel using a Tris-tricine buffer, along with a sample of the blocked enzymes as a control. The gel was transferred to nitrocellulose, and the nitrocellulose was blocked with milk and BSA as described above. Interaction of the secondary antibody with the anti-CAP-3 used to immunoprecipitate was reduced by treating the blot with 2 μg/mL goat anti-rabbit IgG in blocking buffer at room temperature for one hour. The blot was then probed with anti-Ich-2 (1:1000 dilution in blocking buffer), anti-ICE p20 and anti-ICE p10 antibodies, or anti-CPP32, as described above.

Inhibition of caspase amidolytic activity by CAP-3.

Figure 1A-C shows progress curves for the hydrolysis of 20 μM tetrapeptide *p*-nitroanilides by ICE or its homologues in the presence of CAP-3. The progress curves for the inactivation of caspases were fit into the integrated rate equation.

$$P = v_{ss}t + \frac{(v_0 - v_{ss})}{k_{app}}\left(1 - e^{-k_{app}t}\right) \qquad (1)$$

where v_o, v_{ss} and k_{app} represent the initial and steady state velocities and an apparent first-order rate constant for inactivation of the enzyme, respectively (Morrison *et al* (1988) *Adv. Enzymol.* 61:201-301). For ICE (Figure 1A), the best fit was obtained when the steady state velocity was zero, and the variation of k_{app} with CAP-3 concentration for the inactivation is shown in Figure 1D. For CPP32 (Figure 1B), there was no difference in k_{app}, and the curves are essentially superimposable. For Ich-2 (Figure 1C), a more complicated situation arises, as v_{ss} is not zero in the absence of CAP-3. The addition of the CAP-3 results in a more rapid, though still complicated, inactivation.

The substrate concentration used in the previous experiment is approximately equal to K_M for ICE (K_M = 24 μM) and CPP32 (K_M = 10 μM) (21), but much less than K_M for Ich-2

(K_M = 700 μM) (23). At higher substrate concentration (200 μM) the degree of inhibition of ICE (Figure 2A) and Ich-2 (Figure 2B) is reduced. The results from experiments with ICE at a variety of substrate concentrations are summarized in Figure 2C. The apparent first order inactivation rate constant shows a hyperbolic dependence on substrate concentration with an apparent dissociation constant (14 μM) that is similar to the apparent K_M for this substrate determined from the initial rates (11 μM). The results shown in Figures 1 and 2 suggest that CAP-3 is a slow-binding inhibitor of ICE and Ich-2 that binds competitively with substrate, but that CPP32 is only weakly affected by CAP-3.

To measure the rate of inactivation in the absence of any competing active site ligand, such as substrate, as well as to examine the effects of higher concentrations of CAP-3, the serpin analogue was preincubated with the proteases in the absence of substrate, and the reaction mixture was then diluted into the assay to measure remaining activity. The decrease in activity with time is shown in Figure 3. For ICE (Figure 3A), the first order loss of activity was observed, and K_{app}, the rate constant for the inactivation, increases from 1 x $10^{-4}s^{-1}$, when no serpin is present, to 2.3 x $10^{-3}s^{-1}$, in the presence of 5 μM CAP-3. Figure 3D shows that the variation of k_{app} with CAP-3 concentration displays saturation kinetics, with an apparent dissociation constant of 3.2 $\pm$ 0.6 μM.

For CPP32, (Figure 3B), no serpin-dependent decrease in proteolytic activity was observed. With Ich-2 (Figure 3C), a complicated pattern of inactivation was observed. In the presence of zero or one equivalent of CAP-3, a biphasic loss of activity was observed: a rapid phase with a rate constant of about 2 x $10^{-3}s^{-1}$ and a slow phase with a rate constant of about 2 x $10^{-4}s^{-1}$. When two (not shown) or four equivalents of CAP-3 were added, the slow phase was not observed, and only a rapid phase with a rate constant of about 2 x$10^{-3}s^{-1}$ was seen. This rate is about four times faster than the rate of inactivation of ICE with an equivalent amount of CAP-3. A small slow phase of hydrolysis would not be detectable in this experiment, as the rate of substrate hydrolysis by Ich-2 was low compared to that by ICE. Thus, activity less than about 10-20% of control activity can not be detected due to background hydrolysis. Due to the complex behavior exhibited by Ich-2 in the absence of CAP-3 in the experiments depicted in Figures 1 and 3, the interaction of CAP-3 with Ich-2 was not analyzed kinetically in greater detail. Based on these results, the order of affinities of the different enzymes for CAP-32 appears to be ICE $\sim$ Ich-2$_{>>}$CPP32.

No evidence for recovery of ICE activity could be seen, even with prolonged (5 hr.) incubation of the diluted ICE-CAP-3 complex with substrate. Consistent with earlier observations (Sprecher *et al* (1995) *J. Biol. Chem.* 270:29854-29861), control experiments showed no inhibition of the non-ICE-related cysteine proteinases papain and cathepsin B upon preincubation with CAP-3 in the absence of substrate (data not shown).

Isolation of the enzyme-CAP-3 complexes.

Since serpins are known to form SDS-stable complexes with cysteine proteinases (Potempa *et al* (1994) *J. Biol.* Chem. 269:15957-15960), we attempted to detect complex formation between ICE and CAP-13 by SDS-PAGE. Electrophoresis followed by silver staining or immunoblotting showed no evidence for formation of a high molecular weight complex (data not shown). CAP-3 contained a small, variable amount of a species migrating on SDS-PAGE at 38 kDa, consistent with cleavage at the putative specificity-determining residue in the reactive site loop. Incubation with ICE or Ich-2, but not CPP32, caused the intensity of this band to increase in a time-dependent manner, but the total amount of this component never exceeded about 10% of the total CAP-3. The behavior of CAP-3 in this system is similar to the behavior of CrmA with ICE in a similar experiment (Komiyama *et al,* (1994) *J. Biol. Chem.* 269:19331-19337).

To isolate the serpin-caspase complex, *in vitro* immunoprecipitation was performed. As a control to verify that the interaction between caspase and CAP-3 requires a free active site in the caspase, the active site cysteines of ICE or Ich-2 were alkylated using the irreversible ICE inhibitor biotin-Tyr-Val-Ala-Asp-acyloxymethylketone (Thornberry *et al,* (1994) *Biochemistry* 33:3934-3940). An assay with 200 μM AcYVAD-pNA showed that 10% of the original ICE activity and 7% of the original Ich-2 activity remained. Using protein A-agarose, a mixture of active site-blocked or untreated ICE or Ich-2 with CAP-3 was precipitated with anti-CAP-3 antibodies. After SDS-PAGE on a 10-20% polyacrylamide gradient gel using a Tris-tricine buffer system and immunoblotting, a band that was immunoreactive with anti-p10 could be detected in only the ICE (Figure 4A) or Ich-2 (Figure 4B) reactions that contained untreated caspase. No band was seen in control reactions without added CAP-3. A faint band was detectable from the reactions with blocked enzyme with an intensity approximately corresponding to the amount of activity remaining in the blocked enzyme samples. The p20 subunits comigrates in this gel system with a minor

immunoreactive component of the anti-CAP-3 antibodies used in the immunoprecipitation. Thus, a small amount of p20 would be obscured by this impurity. The immunoprecipitates were separated on a 14% polyacrylamide gel using a Tris-glycine buffer system and p20 was more clearly resolved from this impurity to give the same result as that seen with p10 (data not shown). An analogous experiment using CPP32 showed no evidence for precipitation of a CPP32-CAP-3 complex (data not shown). To ascertain that blocking the active site does not interfere with recognition by the anti-ICE or anti-Ich-2 antibodies, a standard sample of blocked enzyme was loaded directly onto the gel. These results show that a tightly bound complex between ICE or Ich-2 and CAP-3 forms when the two proteins are incubated together *in vitro*, which is precipitated with anti-CAP-3 antibodies. In addition, blocking the active site of the enzyme with a covalent inactivator greatly diminishes formation of the complex.

The serpin analogue CAP-3 is unique among human serpin analogues in that it has an acidic residue in the putative specificity-determining position of the reactive site loop. As the above results show, CAP-3 inhibits the amidolytic activity of several caspases. Hydrolysis of peptide substrates by interleukin-1β-converting enzyme and Ich-2 is inhibited by CAP-3 in a time-dependent manner. The reaction of interleukin-1β-converting enzyme with CAP-3 shows a hyperbolic dependence on the concentration of CAP-3, and high concentrations of peptide substrate protects against this inactivation. This is consistent with a two-step kinetic model for inactivation. Hydrolysis of tetrapeptide substrate by CPP32 is not inhibited by CAP-3. The complex of interleukin-1β-converting enzyme or Ich-2 with CAP-3 can be immunoprecipitated, but no complex with CPP32 can be detected. No complex can be immunoprecipitated if the active site of the caspase is blocked with a covalent inhibitor. These results show that CAP-3 is an inhibitor of interleukin-1β-converting enzyme and of its close relative Ich-2, but not of the more distantly related CPP32. CAP-3 is the first example of a human serpin analogue that inhibits members of this class of cysteine proteinases.

All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications, patents and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporate by reference.

The invention now having been fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

What is claimed is:

1. A method for treating a disease or symptoms of a disease mediated by a caspase in an individual in need thereof, said method comprising:

administering to said individual a composition comprising a gene coding for an intracellular mammalian serpin in an amount sufficient to inhibit activity of said caspase upon transient expression of said gene in a target tissue affected by said disease, whereby said disease or said symptoms are treated.

2. The method according to Claim 1, wherein said caspase is selected from the group consisting of caspase-1, caspase-3, caspase-4 and caspase-8.

3. The method according to Claim 1, wherein said intracellular mammalian serpin is CAP-3.

4. The method according to Claim 1, wherein said target tissue is selected from the group consisting of heart, liver, brain, and lung.

5. The method according to Claim 1, wherein said target tissue is a xenotransplant tissue.

6. A method for decreasing inflammation mediated by caspase-1 in an individual in need thereof, said method comprising:

administering to said individual a composition providing a gene coding for an intracellular mammalian serpin in an amount sufficient to inhibit activity of said caspase-1 upon transient expression of said gene in an inflammed target tissue, whereby said inflammation is decreased.

7. The method according to Claim 6, wherein said inflammed target tissue is the liver of said individual.

8. The method according to Claim 7, wherein said administering is intravenously afferent to said liver.

9. The method according to Claim 6, wherein said inflammed target tissue is a brain tissue of said individual.

10. The method according to Claim 9, wherein said administering is intrathecally into said brain tissue.

11. The method according to Claim 6, wherein said inflammed tissue is heart tissue of said individual.

12. The method according to Claim 11, wherein said administering is via a catheter into said heart tissue.

13. The method according to Claim 6, wherein said inflammation is chronic inflammation.

14. A method for modulating apoptosis mediated by a caspase-4 in an individual in need thereof, said method comprising:

administering to said individual a composition providing a ligand which binds to a transcription product or an expression product of a nucleotide sequence comprising a gene coding for an intracellular mammalian serpin in an amount sufficient to alter activity of said caspase-4 in an apoptotic target tissue, whereby apoptosis in said target tissue is modulated.

15. The method according to Claim 14, wherein said activity of said caspase-4 is inhibited.

16. The method according to Claim 15, wherein said apoptotic target tissue is a liver in acute failure.

17. The method according to Claim 16, wherein said acute failure is due to exposure to one or more toxins, cirrhosis or infection with a hepatitis virus.

18. The method according to Claim 14, wherein said apoptotic target tissue is selected from the group consisting of heart, liver, brain and lung.

19. A method for treating a lung disease, symptoms of which are mediated by a caspase, in an individual in need thereof, said method comprising:

administering to the lungs of said individual a composition comprising a gene coding for an intracellular mammalian serpin in an amount sufficient to inhibit activity of said caspase upon transient expression of said gene in said lungs, whereby said lung disease is treated.

20. The method according to Claim 19, wherein said lung disease is silicosis.

21. The method according to Claim 19, wherein said symptoms include emphysema.

22. A method for treating a neurodegenerative disease mediated by a caspase in an individual in need thereof, said method comprising:

administering to said individual a gene coding for an intracellular mammalian serpin in an amount sufficient to inhibit activity of said caspase upon transient expression of said gene in a brain tissue affected by said neurodegenerative disease, whereby further neurodegeneration is prevented or diminished.

FIG 1A

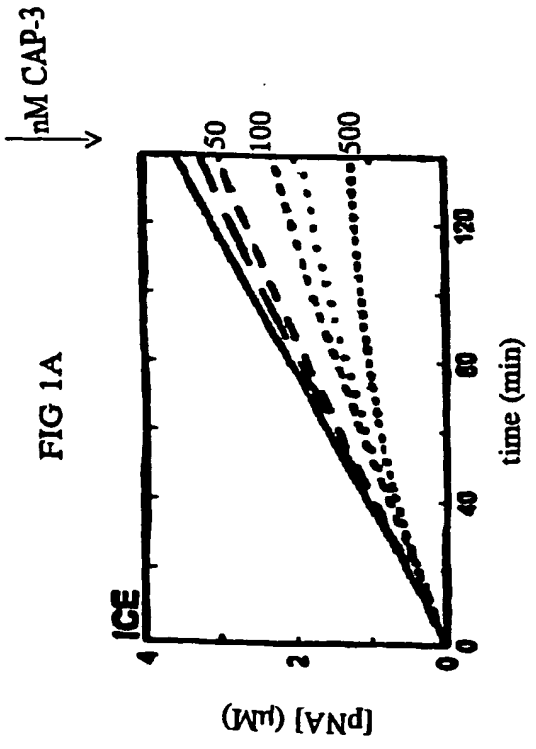

FIG 1B

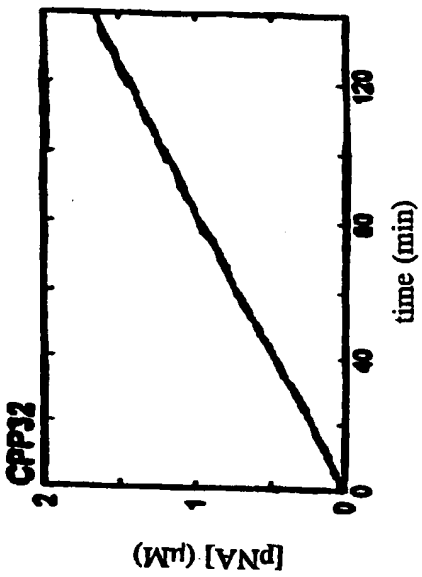

FIG 1C

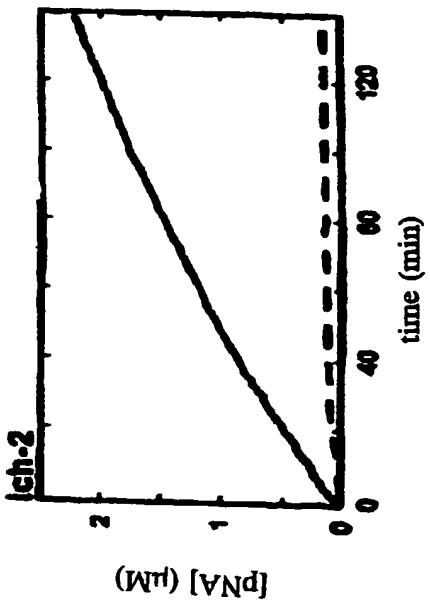

FIG 1D